The enzymology of itaconic acid production in Aspergillus terreus (NRRL 1960).

Grayson, Steven

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THE ENZYMOLOGY

OF ITACONIC ACID PRODUCTION IN

ASPERGILLUS TERREUS (NRRL 1960)

Submitted by Steven Grayson
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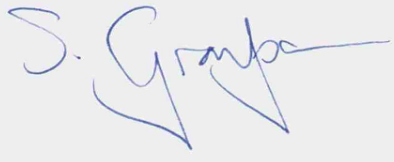
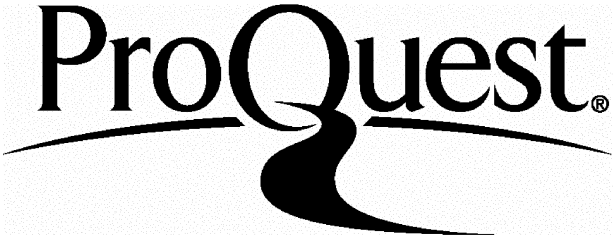
University of Bath
1985





ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisor Prof. P.D.J. Weitzman for his unfailing forbearance, encouragement and enthusiasm throughout the past three years. I am also grateful to Drs. P. Senior and P. Rodgers (Imperial Chemical Industries, Agricultural Division, Billingham) and to Dr. R. Otto (University of Bath) for helpful discussions.

ABBREVIATIONS

CAD: *cis*-Aconitate Decarboxylase (EC 4.1.1.6)

DTNB: 5,5'-dithiobis-(2-nitrobenzoic acid)

All other abbreviations used in this thesis are those recommended in the Biochemical Society Publication "Policy of the Journal and Instructions to authors" (*Biochem. J.* (1984) *217*, 1).

CONTENTS

ABSTRACT

The enzymology of itaconic acid (methylenesuccinic acid) production by Aspergillus terreus has been investigated. The organism has been shown to possess many of the enzymes of the citric acid cycle when cultured under conditions which did support, and conditions which did not support, itaconate production. No significant differences in the levels of individual citric acid cycle enzyme activities between the two modes of culture were detected.

A new, more sensitive, assay for itaconate has been devised. An unfractionated extract of Pseudomonas aeruginosa, capable of converting itaconate to pyruvate, provides the basis of the enzyme-linked assay in which the ultimate product, pyruvate, is detected spectrophotometrically as its phenylhydrazone derivative. The assay has been shown to be applicable to the monitoring of itaconate production during the fermentation of Aspergillus terreus.

Furthermore, the enzyme-linked assay has been adapted to operate in a continuous mode and hence to permit measurement of the rate of formation of itaconate by cell-free extracts of Aspergillus terreus. cis-Aconitate decarboxylase (CAD) activity was detected in mycelia harvested in the acid-producing phase of growth, but was not found in mycelia harvested prior to the production of

acid, nor when the organism was grown under conditions which did not support acid production. The enzyme present in the crude extract precipitated between 40% and 60% ammonium sulphate saturation. Studies on this partially purified extract indicated a Km value for cis-aconitate of 0.15mM and a pH optimum of 5.5.

Investigation of Aspergillus terreus grown in batch culture revealed that the depletion of phosphate in the culture medium coincided with a rise in intracellular CAD activity and subsequent itaconate production. In addition, substantial CAD activity was detected in cell-free extracts of Aspergillus terreus even after the production of itaconate had ended, thus suggesting that other factors, which are possibly amenable to external control, may be responsible for the cessation of acidogenesis.

INTRODUCTION TO SECONDARY METABOLISM

The growth profile of a microorganism in batch culture may be divided into three major phases. The lag phase immediately follows inoculation and represents the period in which the organism makes the necessary metabolic modifications for growth in the new environment. This is followed by the exponential, or unrestricted, growth phase which continues for as long as all substrates necessary for growth are present in excess, and no inhibitors are present. The final phase is the stationary phase in which growth has stopped, usually because of the exhaustion of a growth-essential nutrient from the culture medium. Frequently, the exponential growth phase and the stationary phase are separated by a period of decelerating growth in which an essential substrate reaches a growth-limiting concentration. These three phases were first delineated by Lane-Claypon (1909).

While it was recognized that the exponential growth phase was associated with extensive metabolic activity, involving rapid synthesis and replication of cellular constituents, it was originally believed that there was almost complete metabolic inactivity during the

stationary phase. However, during the 1920s and 1930s, the structures of many metabolites isolated from microbial culture media were elucidated. Particular interest was taken in those metabolites isolated from stationary-phase cultures of filamentous fungi. It was soon realised that a large proportion of these compounds, of varying structural complexity, had no apparent metabolic function in the organism. Using a term borrowed from plant physiologists, they were named 'secondary products of metabolism'. These 'secondary metabolites' were shown to be produced at the end of the exponential growth phase and during the stationary phase. Compounds essential for the growth of the organism were known as 'primary metabolites'.

Bu'Lock, who has pioneered much of this work (for reviews, see Bu'Lock, 1961, 1965, 1967 and 1975), has also introduced new terms to describe the second and third phases. 'Trophophase' describes the growth phase in which the cells synthesise primary metabolites; 'idiophase' describes the period characterized by metabolic idiosyncrasies in which the secondary metabolites are produced.

The filamentous fungi have proved to be particularly prolific in their secondary metabolites. A recent classification of secondary metabolites (Turner, 1971) listed over one thousand different compounds. Only a few

of these are known to be produced by more than three or four species and, since only a minority of species have so far been investigated, it is confidently predicted that the range of products is far wider than has so far been revealed.

Secondary metabolism is an enigma. Comparative biochemistry deals with the 'unity of biochemistry' and sets out to elucidate the differences in primary metabolism between major taxonomic groupings. However, secondary metabolites characterize sub-species, species, or groups of species and are, therefore, genotypically specific. Furhermore, in many cases they are also phenotypically specific, in that their production is extremely sensitive to culture conditions and previous history (Bu'Lock, 1975). Such an example is provided in this report.

The great diversity and eccentric distribution of fungal secondary metabolites has been described as being "biochemically bizarre" (Weinberg, 1974), "an extraordinary bestiary of organic compounds" (Weinberg, 1970), while Bu'Lock (1961) resorted to poetic imagery:

> "While the enzymologist's garden is a dream of uniformity, a green meadow where the cycles of Calvin and Krebs tick round in disciplined order, the organic chemist walks in an untidy jungle of uncouthly named extractives, rainbow displays of pigments, where in every bush there lurks the mangled shape of some new alkaloid, the exotic perfume of some new turpentine, or some shocking and explosive polyacetylene."

SECONDARY METABOLISM AND METABOLITE OVERPRODUCTION IN GENERAL

Not all molecules which are excreted into the culture media of microorganisms are characterized by their apparent lack of function in cell growth and development. Although the commercial importance of many secondary metabolites has been the prime reason for rapid advances in the investigation of their production, it is the interest of the industrialist in any commercially-important biochemical that has helped to demonstrate the additional existence of primary metabolite overproduction processes. Industrialists tend to treat metabolite overproduction as a phenomenon in itself, whereas the research scientists usually separate the production of secondary metabolites, such as antibiotics, from the over-synthesis of primary metabolites, such as citric and gluconic acids. However, it is becoming increasingly apparent that secondary metabolites are not without function to the organism, and it is possible that although primary and secondary metabolites fulfill different roles in the survival of the organism, the process of differentiation which leads to their excretion may be stimulated by common physiological effectors.

Demain (1983) has proposed two reasons for the reluctance to accept that antibiotics are ecological effectors:

firstly, antibiotics are of major economic significance, and patents on them are more easily obtained if they are not considered products of nature; secondly, experiments to prove their ecological significance are more often unsuccessful than successful. The fact that secondary metabolites of green plants have never been doubted of performing valuable functions is indicative of some bias against those of microorganisms; furthermore, on the second point, recent studies of metabolite-producing fungi in their natural habitats have provided further evidence in support of the competitive advantage conferred by secondary metabolite production (Jarvis et al., 1981; Calam, 1982).

The increasing acceptance of the view that secondary metabolites do fulfill useful roles has led to suggestions that, in addition to some acting as antagonistic agents, others may serve as symbiotic agents, sexual hormones, effectors of sporulation and germination, and metal transporters (Demain, 1983). Another theory, which puts more emphasis on the process rather than the product, is the maintenance hypothesis of Bu'Lock (1961), the view that the operation of secondary metabolism keeps cellular metabolism in working order when growth is not possible.

However, the current line of thought arises from a synthesis of these different theories. It is possible to

envisage a situation in which restrictions on the growth of the organism lead to metabolic compensatory actions, some of which simply serve to maintain primary metabolism as best as possible, some of which lead to morphological differentiation in order to survive, and possibly move away from unfavourable conditions, and some of which have the additional advantage of deterring predators. Moreover, since coining the terms 'trophophase' and 'idiophase', Bu'Lock (1975) has emphasized that a well-marked division between growth-directed and secondary metabolic activities will become less useful when a better understanding of these activities is attained. The importance of this becomes especially apparent when it is observed that, in many cases, the inability to decide whether a metabolite is produced during the idiophase or the trophophase, or during both, tends to create needless confusion.

As Bu'Lock (1975) has pointed out, in unlimited growth the rate of uptake of substrates into the mycelium, and the rate at which they are used for mycelial development, are governed by intrinsic properties of the mycelium and are, therefore, as high as they possibly can be for the system. Unlimited growth is, therefore, intrinsically balanced in that the relative contributions of individual processes remain constant, and consequently it is truly replicatory. Under such conditions, it is unlikely that metabolites are overproduced. In batch culture, as the

progressive consumption of substrate carries the organism into and through a spectrum of increasingly limited growth, the growth becomes unbalanced and differentiation of the organism occurs. Thus, "secondary metabolism is an aspect of the differentiation which limited growth implies" (Bu'Lock, 1975). Whether differentiation takes the form of the overproduction of a so-called primary or secondary metabolite depends on the organism and the severity of growth limitation. In this way, the same physiological effect can be seen to lead to the overproduction of, for example, either an organic acid of fundamental metabolic importance to the organism, or an apparently useless molecule, the ecological role of which has yet to be realised.

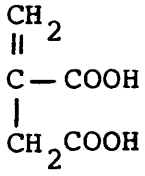
It is also important at this stage to distinguish between the products of unbalanced growth, described above, and natural products of balanced (unrestricted) growth. In an aerobically-grown organotrophic organism, the latter consist only of carbon dioxide, water, biomass and heat. However, under conditions of anaerobic growth, the organism uses an organic compound as the ultimate electron acceptor as well as the electron donor. The products of such 'fermentations' include the commercially important molecules ethanol, acetate, lactate, acetone and butanol.

A qualification to be added here, however, is the

discovery of metabolite overproduction in fully aerobic, carbon-sufficient, nutrient-limited chemostat cultures of facultative anaerobic bacteria (Neijssel and Tempest, 1979; Teixeira De Mattos et al., 1982) in which growth and energy production are seen to be uncoupled. In such cases, it is proposed that the organism uses the reduction of an organic compound, the product of which is subsequently excreted, to regenerate the oxidised form of essential reducing equivalents so that metabolism may continue. Such a discovery does not contradict the hypothesis, proposed earlier, in which metabolite overproduction is absent in unrestricted growth, because it can be argued that a growth restriction is essential for the attainment of steady-state growth in a chemostat. However, the results of these discoveries do serve as an example of the potential use of chemostat cultures in the study of the factors affecting metabolite overproduction and in the possible elucidation of conditions for optimum product formation.

ITACONIC ACID PRODUCTION BY ASPERGILLUS TERREUS

The microbial production of itaconic acid provides a good example of the need to move away from classifying overproduced compounds as either 'primary' or 'secondary' metabolites. Itaconic acid (methylenesuccinic acid) has the following structure:



The close structural similarity between this compound and intermediates of the citric acid cycle, and its relative simplicity compared to the large size and complex structure of other secondary metabolites, encourages its recognition as a primary product of metabolism. On the other hand, the absence of any apparent metabolic function means that it may be regarded as a typical secondary metabolite. There is, therefore, a need to treat the microbial production of itaconate in isolation from other such processes until similarities between the biochemical mechanisms of overproduction are made apparent.

Commercial Importance of Itaconate

Itaconate has various uses in the paper, paint, textile and plastics industries. Esters such as dimethyl

itaconate and dibutyl itaconate may be polymerized or copolymerized with other monomers such as methacrylates, styrene or vinyl chloride, to form a wide variety of plastics of considerable interest. However, the availability of less expensive starting materials such as maleic and fumaric acids has prevented more widespread use of itaconate in the plastics industry. According to Miall (1978), over three-quarters of the itaconate manufactured is used in styrene butadiene copolymers and for lattices and emulsions in general. The most important use of these is in carpet backing, with a significant proportion employed for paper coating. Most of the remainder is used in acrylonitrile copolymers for synthetic fibre manufacture. Inclusion of about 5% itaconate in the polymer imparts the ability to take and hold printing inks. Pfizer is probably the largest manufacturer of itaconate. Melle-Bezons has the only other plant in Western Europe, and itaconate is also made in Russia and Japan. No published information is available on sales volume.

Historical Background

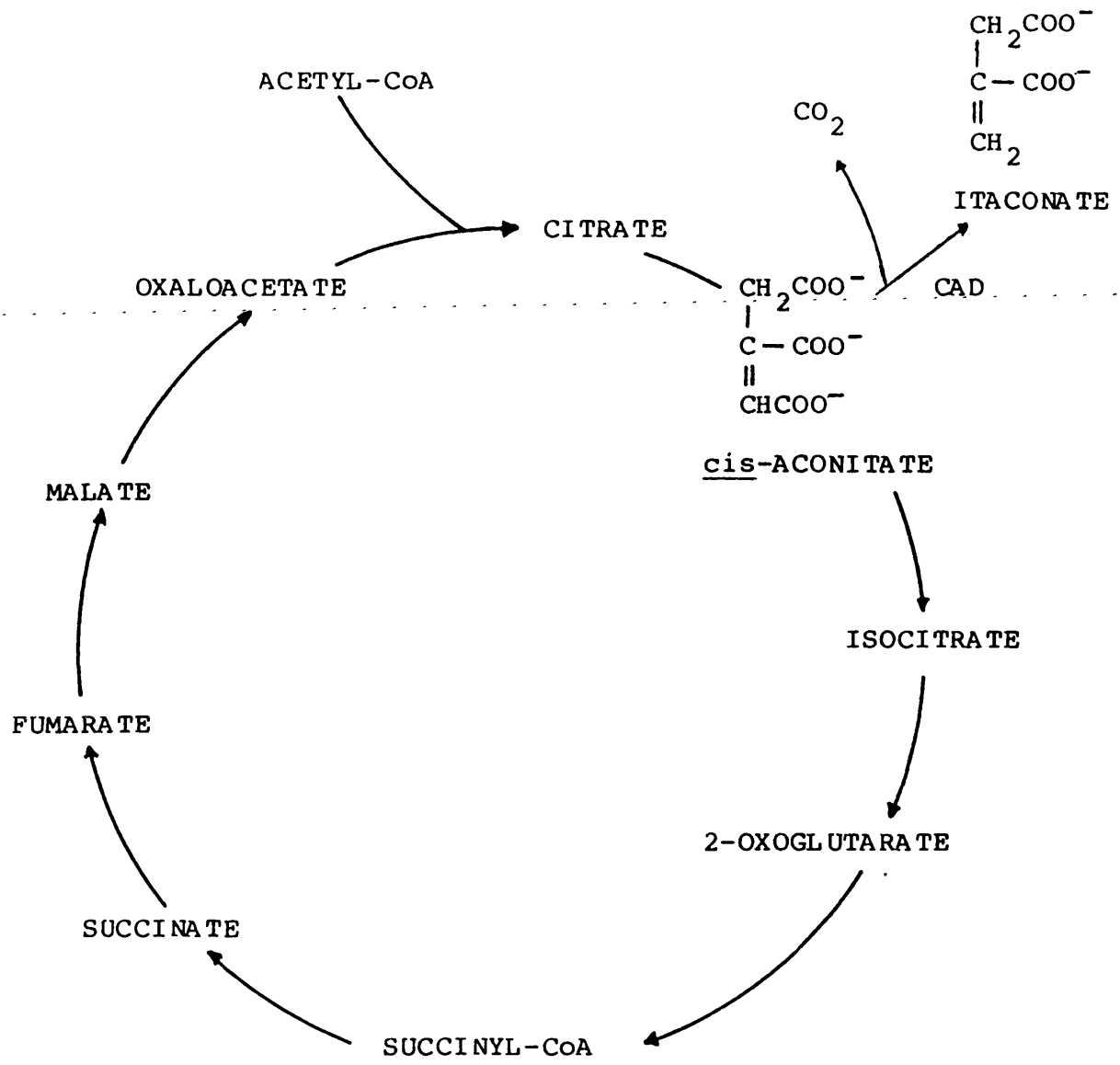
Itaconic acid was originally obtained by pyrolysis of citric acid. Loss of water from citric acid results in the formation of aconitic acid and, on subsequent decarboxylation, two isomeric compounds were produced: the anhydrides of itaconic and citraconic acids. However, the process was not commercially successful.

Aconitic acid is present in sugar cane juice and was believed to interfere in sucrose crystallization. Removal of calcium aconitate by sugar refiners, and its conversion to itaconic acid by heating, was the principal source of itaconic acid until the fermentation process was developed.

Itaconate was first reported as a fungal metabolite by Kinoshita (1931) who described the new species Aspergillus itaconicus. Calam et al. (1939) later showed itaconate also to be a product of certain strains of Aspergillus terreus. Subsequent screening of thirty cultures of Aspergillus terreus by Moyer and Coghill (1945) revealed only one, viz. NRRL 265, which gave promising yields from the point of view of industrial exploitation. Lockwood and Reeves (1945) screened over three hundred strains of Aspergillus terreus, which had been isolated from soil, and found that eleven of these gave a yield from glucose in excess of 72% (w/w). From these screens, two strains (NRRL 265 and NRRL 1960) were investigated more thoroughly, and NRRL 1960 was chosen for pilot-scale production of itaconate. This strain, or mutants derived from it, is believed to be the organism used in all subsequent manufacturing processes, and has consequently been the subject of thorough nutritional and physiological investigation (for reviews, see Smith et al., 1974; Lockwood, 1975, 1979; Miall, 1978).

Biochemistry of Itaconate Production

Kinoshita (1931) originally proposed the route of itaconate formation in Aspergillus terreus to be via the decarboxylation of the citric acid cycle intermediate, cis-aconitate. The cis-aconitate decarboxylase (CAD) reaction is shown in Scheme 1. Bentley and Thiessen (1957) performed the first comprehensive study of the biochemistry of itaconate formation and accordingly produced evidence for the occurrence of CAD in Aspergillus terreus. From experiments with radiolabelled glucose, and the inhibitors fluoride and iodoacetate, these authors concluded that glucose dissimilation occurred via the Embden-Meyerhof-Parnas pathway, a proposal which has not since been disputed. The use of radiolabelled succinate and acetate added further support to the hypothesis of Kinoshita (1931), and the subsequent detection of intracellular 2-oxoglutarate, succinate and malate provided additional evidence for the operation of the citric acid cycle in the organism. These authors also reported the manometric detection of cis-aconitate decarboxylation, catalysed by a cell-free extract of Aspergillus terreus, and showed the initial rate of this reaction to be slower when citrate and isocitrate were used instead of cis-aconitate. From this they proposed that cis-aconitate was a more immediate precursor of itaconate in the organism and that conversion of citrate to itaconate occurred under the successive action of aconitase and CAD.

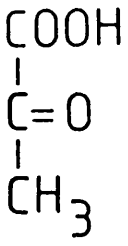


SCHEME 1: THE CITRIC ACID CYCLE SHOWING THE cis-ACONITATE DECARBOXYLASE (CAD) REACTION

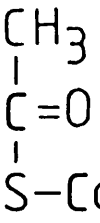
These results contradict those of Eimhjellen and Larsen (1955) who have reported that fluorocitrate (an inhibitor of aconitase) led to a stimulation of itaconate production from both citrate and glucose. Conversely, the results obtained by Corzo and Tatum (1953), who used radiolabelled acetate, were in accordance with those of Bentley and Thiessen (1957).

Lal and Bhargava (1962) studied the effect of fluoride inhibition on the production of itaconate by Aspergillus terreus and its reversal by pyruvate. These authors showed that fluoride inhibited a step in the conversion of pyruvate to itaconate more strongly than it inhibited enolase. Arguing that all known enzymes on the proposed pathway from pyruvate to itaconate (pyruvate dehydrogenase, citrate synthase, aconitase and CAD) were less sensitive to fluoride than was enolase, they suggested that some other pathway must operate. Shimi and Nour El Dein (1962) proposed a condensation of acetate and succinate to form 1,2,3-propanetricarboxylic acid, which could then be dehydrogenated and decarboxylated to itaconate.

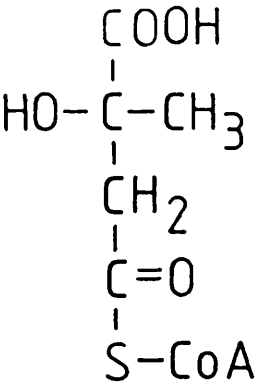
Nowakowska-Waszczuk (1973) reported finding no evidence of an active citric acid cycle in Aspergillus terreus NRRL 1960, or in two high-yielding mutants derived from it. Mitochondria extracted from these strains oxidised



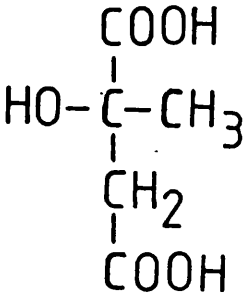
Pyruvic Acid



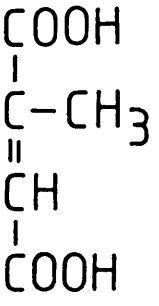
Acetyl-CoA



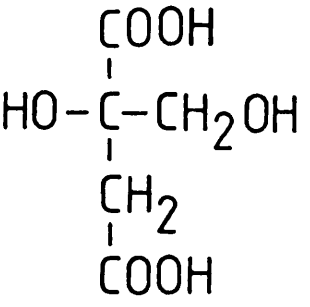
Citramalyl-CoA



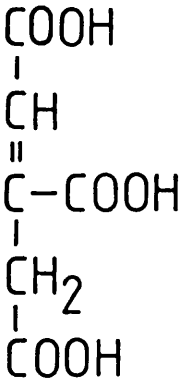
Citramalic Acid



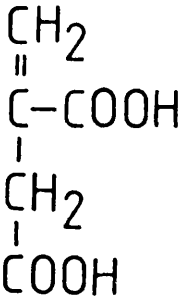
Citraconic Acid



Itatartaric Acid



Aconitic Acid



Itaconic Acid

reduced pyridine nucleotides with good respiratory control, but did not oxidise citric acid cycle intermediates either in the presence of ADP or cytochrome c, nor after disruption of the mitochondrial membrane. It was suggested that itaconate was not synthesized via the citric acid cycle.

Smith *et al.* (1974) suggested the following series of reactions may occur:

cis-aconitate ⟶ citraconate ⟶ citramalate ⟶ itaconate;

Jakubowska (1977) favoured the following scheme:

pyruvate + acetyl-CoA ⟶ citramalyl-CoA ⟶ citramalate ⟶ itaconate;

and Lockwood (1979) suggested another route:

pyruvate + acetyl-CoA ⟶ citramalate ⟶ citraconate ⟶ itatartrate ⟶ itaconate.

Chemical structures are shown on the facing page.

AIMS OF THIS PROJECT

The alternative routes for the formation of itaconate in Aspergillus terreus, shown on the previous page, were proposed primarily because of the accumulated evidence contradicting the existence of CAD.

The failure of Nowakowska-Waszczuk (1973) to find any evidence for the operation of the citric acid cycle was intriguing. This was especially so because of the generally accepted central role of the citric acid cycle in metabolism. Nowakowska-Waszczuk (1973) had cultured the organism using sucrose as the sole source of carbon and it would therefore be expected that synthesis of many of the amino acids essential for protein formation would occur via such citric acid cycle intermediates as succinyl-CoA, 2-oxoglutarate and oxaloacetate. Furthermore, synthesis of porphyrins, precursors of haem proteins, would be expected to occur via succinyl-CoA. Thus, with a carbohydrate carbon source, most enzymes of the cycle would be essential for growth.

The primary aims of the project were, therefore, to make a closer analysis of this area of metabolism in

itaconate-producing Aspergillus terreus. It was considered necessary to look for the presence of the enzymes of the citric acid cycle and CAD itself. It was hoped that the original hypothesis of Kinoshita (1931) would be either confirmed or repudiated. During the course of these investigations, independent evidence which contradicted the results of Nowakowska-Waszczuk (1973) was published (Winskill, 1983), but because of the similarity of the conclusions drawn by this author and the results presented here, detailed discussion is postponed until later sections.

MATERIALS AND METHODS

ORGANISMS

Aspergillus terreus (NRRL 1960, ATCC 10020) was obtained from the Commonwealth Mycological Institute, Kew, U.K. Wild-type Pseudomonas aeruginosa (PA01) was from Professor Weitzman's culture collection at the University of Bath, and a culture of isocitrate lyase-deficient Pseudomonas aeruginosa (PAC 501, see Skinner and Clarke, 1968) was kindly provided by Professor P.H. Clarke, Department of Biochemistry, University College, London, U.K.

REAGENTS

Itaconic acid, cis-aconitic acid, ATP and phenylhydrazine hydrochloride were from Sigma Chemical Co., Poole, Dorset, U.K. Coenzyme A and oxaloacetic acid were from Boehringer, Mannheim, Germany, and all other substrates, cofactors and enzymes for the enzyme assays were from Sigma. Sucrose, ammonium sulphate, calcium chloride, magnesium sulphate and potassium dihydrogen orthophosphate, used for the routine culturing of Aspergillus terreus, were from B.D.H. Chemicals, Poole, Dorset, U.K. and were all analytical grade. All other chemicals used were analytical grade.

MEDIA

Czapek-Dox liquid medium was from Oxoid Ltd., London, U.K. The constituents were (l^{-1} distilled water): sucrose (30g), $NaNO_3$ (2g), KCl (0.5g), magnesium glycerophosphate (0.5g), K_2SO_4 (0.35g) and $FeSO_4$ $7H_2O$ (0.01g). Czapek-Dox agar contained additionally 15g of Bacto-Agar from Difco Laboratories, Detroit, U.S.A. Potato-carrot agar was prepared in this laboratory. Diced potato (20g) and carrot (20g) were boiled in 500ml of distilled water for 30min. The residual potato and carrot were removed by filtration through muslin and the filtrate was added to 15g of Bacto-Agar and then made up to one litre.

SEPHADEX G-25 GEL-FILTRATION

Desalting, after the ammonium sulphate fractionation, and the removal of other low molecular weight molecules from enzyme extracts, were routinely performed by gel-filtration using Sephadex G-25 (PD-10) columns obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. The columns had a bed volume of 9ml and a bed height of 5cm. The sample (2.5ml) was loaded onto the column and the initial 2.5ml of eluate was discarded. The high molecular weight components were then eluted with 3.5ml of buffer.

CULTURE CONDITIONS

Aspergillus terreus

Stock cultures of Aspergillus terreus were maintained on potato-carrot agar slopes under sterilized paraffin oil at 4°C, and transferred to Czapek-Dox agar slopes, incubated at 32°C, for spore production. Conical flasks (500ml), each containing 100ml of medium, were inoculated with spores washed from 7-day old Czapek-Dox slopes using 0.85% saline solution and were incubated on a rotary shaker at 32°C. In later experiments, where indicated, a 5-day old culture from such a flask was used as the inoculum (2% v/v) for the fermenter and replicate flask cultures. As indicated in the Results section (p.35), high yields of itaconate were produced consistently when Aspergillus terreus was cultured in a medium containing 10% sucrose (or glucose) and tap water. The constituents were (l^{-1} tap water): sucrose (100g), $(NH_4)_2 SO_4$ (3g), $CaCl_2 2H_2O$ (2g), $MgSO_4 7H_2O$ (0.5g) and KH_2PO_4 (0.1g); the final pH was adjusted to 3 using 2M HCl. Mycelia from which preparations of CAD were made were routinely harvested 90h after inoculation. Non-itaconate-producing cultures of Aspergillus terreus were obtained by substituting KNO_3 (3g) for the ammonium sulphate (see p.35 and Nowakowska-Waszczuk, 1973).

The fermenter consisted of a section of QVF glass pipe (20cm long, 16cm diameter) containing 4 stainless steel

baffles, closed at both ends by stainless steel plates, and sealed by rubber gaskets. The impeller was a horizontally mounted turbine (5cm diameter) operating at 500rpm. Continuous monitoring of pH was performed with a recording pH meter in conjunction with a steam sterilizable glass combined electrode (Pye Ingold). Compressed air, at a flow rate of 1 litre min^{-1}, was passed through a sterile multifibre filter and into the fermenter through five 1mm holes in a sparger situated beneath the impeller. The temperature was maintained at $32^{o}C$ by means of a heating coil and cold water cooling finger. A silicone antifoaming reagent was used in the automatic foam control.

Pseudomonas aeruginosa

Both strains of Pseudomonas aeruginosa (PA01 and PAC 501) were maintained and cultured in the same way. The organisms were maintained on nutrient-agar slopes at $4^{o}C$ and grown in 500ml conical flasks, containing 200ml of medium, on a rotary shaker at $32^{o}C$. The medium consisted of sodium-potassium phosphate buffer, pH 7.2 (50mM), NH_4Cl (50mM), disodium itaconate (20mM) and salts (l^{-1}): $CaCl_2\ 6H_2O$ (40mg), $MgSO_4\ 7H_2O$ (80mg), $MnSO_4\ 4H_2O$ (4mg) and $FeSO_4\ 7H_2O$ (4mg).

PREPARATION OF EXTRACTS

Aspergillus terreus

Two methods were used for the preparation of cell-free extracts of Aspergillus terreus. The first was performed for the preparation of extracts from which the activities of citric acid cycle enzymes were determined, and involved the use of the Biogen X-press. In the second method, a mortar, pestle and acid-washed sand were used. In both cases, the mycelium was separated from the culture medium by filtration through muslin and washed with approximately 5 volumes of distilled water followed by 3 volumes of the extraction buffer. The mycelium was then resuspended in an equal volume of the extraction buffer.

X-PRESS EXTRACTION. Three different types of extraction buffer were used for the determination of citric acid cycle enzyme activities. In this way, errors due to the instability of certain enzymes in particular buffers were reduced. The buffers were: (a) 100mM Tris-HCl, 1mM EDTA, pH 8; (b) 100mM HEPES (N-2-Hydroxyethylpiperazine-N-2 -ethanesulphonic acid), adjusted to pH 7.5 with 2M NaOH; and (c) 50mM sodium-potassium phosphate, 3mM $MgCl_2$, 0.1mM EDTA, 150μM phenylmethylsulphonyl fluoride, 5mM mercaptoethanol, pH 7.5. The mycelial suspension was frozen inside the chamber of the X-press and forced through a hole (2mm diameter) by a piston under the action of a hydraulic press. It was passed through the

hole a further three times, and then allowed to thaw slowly. The supernatant resulting from subsequent centrifugation at 40 000g for 20min was the extract used in the assays, and contained between 4 and 5mg of soluble protein ml^{-1}.

MORTAR AND PESTLE EXTRACTION. This method was adopted for the fermentation time-course experiments for which a more rapid extraction procedure was necessary. After being resuspended in the extraction buffer (20mM sodium-potassium phosphate buffer, pH 7.0, for studies on CAD), the mycelium was ground vigorously in a mortar and pestle with acid-washed sand for 2-3 min. The supernatant resulting from subsequent centrifugation at 40 000g for 20 min contained between 1.5 and 4mg of protein ml^{-1}, depending on the age of the mycelium.

Pseudomonas aeruginosa

After 24h growth, cells were harvested by centrifugation at 10 000g for 10min, washed, and resuspended in 20mM sodium-potassium phosphate buffer, pH 7.0. The suspension was then subjected to the output of an Ultrasonics cell disintegrator, operating at 50W, for 2min, with cooling, and centrifuged at 40 000g for 20min. The resulting supernatant, used for the enzymic conversion of itaconate to pyruvate, was found to be stable when frozen; an extract stored at $-15^{o}C$ for 3 months lost only 8% of its initial activity.

AMMONIUM SULPHATE FRACTIONATION

The cell-free extract of Aspergillus terreus was routinely fractionated for the purpose of discontinuous CAD studies. To each 10ml of the unfractionated extract were added 2.43g of solid ammonium sulphate, slowly, with stirring (40% saturation). After 30min, the resulting precipitate was removed by centrifugation at 10 000g for 10min and discarded. To each 10ml of the supernatant were added 1.32g of solid ammonium sulphate (60% saturation) and the mixture was stirred for 30min; after centrifugation (10 000g, 10min), the precipitate was dissolved in 2.5ml of 20mM sodium-potassium phosphate buffer, pH 7.0. This solution of enzyme was then passed down a Sephadex G-25 column (details given on p.21) and the 3.5ml of protein-containing eluate (approximately 10mg of protein) were retained as the partially purified extract.

ANALYSES

Itaconate

Qualitative determination of itaconate was performed according to the chromatographic method of Buch et al. (1952) as adapted by Larsen (1957). Standards of itaconic, cis-aconitic, citric and isocitric acids and a mixture of these (15mg ml^{-1}) were applied (10µl each) to a 25x25cm sheet of Whatman No. 1 paper. Samples of culture media to be analysed were shaken with a proton-charged

cation exchange resin (Dowex 50-X8) before being applied (10µl). The chromatogram was developed using the upper phase of a mixture of ethyl acetate : 4.4M acetic acid (1 : 2) and then sprayed with a solution of 0.04% bromophenol blue in ethanol. The yellow acidic spots were clearly visible against a blue background.

Initially, for the quantitative determination of itaconate, the bromination method of Friedkin (1945) as adapted to spectrophotometry by Adler et al. (1957) was employed. Samples (100µl), containing between 0.1 and 1 mg of itaconate per ml, were added to 100µl of the 'Bromine Reagent' (bromine, 1ml; KBr, 3g; KCl, 1.87g; 1M HCl, 48.5ml; distilled water, to 500ml) and 800µl of 2M HCl in a 1ml cuvette. After 20 min at room temperature, the change in absorbance at 385nm, due to bromine decolourization, was measured spectrophotometrically against a blank which contained water in place of itaconate.

A rapid screening procedure for acid production in different culture media of Aspergillus terreus involved titration of a 5ml aliquot of the medium against 0.1M NaOH. Two drops of a 0.05% solution of phenolphthalein in ethanol : water (1 : 1) was the indicator.

Protein

Protein was measured according to the method of Lowry et

al. (1951) with bovine serum albumin as the standard.

Biomass

Growth was estimated as dry weight; mycelia harvested from the cultures by filtration were washed with distilled water and dried to a constant weight in an oven at 105°C.

Sucrose

Sucrose determinations were performed according to the method of Fairburn (1953), an assay specific for carbohydrates. To the sample (200µl), containing 1 to 10µg of sucrose, was added 1ml of the 'Anthrone Reagent' (0.1% w/v anthrone in 72% sulphuric acid). Following a 10min incubation in a boiling water-bath, and subsequent cooling, the change in absorbance at 620nm was measured against a blank which contained no sucrose.

Ammonia

Ammonia was determined by the phenol hypochlorite method of Mueller-Beissenhirtz and Kellner (1965) as described by Dawes et al. (1971). To the sample (100µl), containing 0.1 to 0.5 µg of ammonia, were added 0.5ml of solution 1 (10g l^{-1} phenol, 0.05g l^{-1} sodium nitroprusside) and 0.5ml of solution 2 (5g l^{-1} NaOH, 0.05g l^{-1} sodium hypochlorite). After 30min incubation at 37°C, the change in absorbance at 625nm was measured against a blank which contained no ammonia.

Phosphorus

The ascorbic acid method of Amman and Hinsberg (1936) as modified by Lowry et al. (1954) and described by Chen et al. (1956) was applied to the determination of phosphorus in the culture media. To the sample (1ml), containing 0.1 to 2 µg of phosphorus was added 1ml of a freshly-prepared mixture of 6N sulphuric acid : distilled water : 2.5% ammonium molybdate : 10% ascorbic acid (1 : 2 : 1 : 1). After a two-hour incubation at 37°C, the change in absorbance at 820nm was measured against a blank which contained no phosphorus.

FAST PROTEIN LIQUID CHROMATOGRAPHY

Ion-exchange chromatography was performed on the Pharmacia FPLC system using the mono-Q column which is an anion exchanger. Partially-purified enzyme extracts were applied and CAD was eluted using gradients of Cl^- as the counterion in 20mM triethanolamine buffer, pH 7.0.

ENZYME ASSAYS

All the enzyme assays, detailed below, were performed in a Pye-Unicam SP8-400 dual-beam spectrophotometer at 25°C. In each case, the reference cuvette contained all the reactants listed except the substrate indicated as the reaction initiator. Initial rates of reactions were shown to be directly proportional to the amount of extract added, over the range used, and activities were shown to be lost on heating the enzyme extract to 100°C for 5min, and cooling, prior to addition. The absorption coefficients employed in the calculations were (l mol^{-1} cm^{-1}): NAD(P)H, $6.2x10^3$; reduced DTNB, $13.6x10^3$; 2,6-dichlorophenolindophenol (DCPIP), $16.1x10^3$; and fumarate, $1.3x10^3$.

Citrate Synthase

Citrate synthase was assayed by the method of Srere *et al*. (1963) in which the CoA produced in the reaction reduces DTNB to form the yellow thionitrobenzoate anion. The 1ml assay mixture contained (final concentrations): 20mM Tris-HCl buffer, pH 8, 1mM EDTA, 0.2mM disodium oxaloacetate, 0.15mM acetyl-CoA, 0.1mM DTNB and enzyme extract. The reaction was started by addition of the oxaloacetate and was followed at 412nm. Acetyl-CoA was prepared from CoA and acetic anhydride by the method of Stadtman (1957).

Aconitase

Aconitase was measured by the enzyme-linked method in which the isocitrate formed from the added substrate, citrate, is converted to 2-oxoglutarate under the action of NADP-dependent isocitrate dehydrogenase with concomitant formation of NADPH. The 1ml assay mixture contained (final concentrations): 20mM Tris-HCl buffer, pH 8, 1mM EDTA, 10mM $MgCl_2$, 0.5mM $NADP^+$, 0.2 units of porcine NADP-dependent isocitrate dehydrogenase, 5mM trisodium citrate and enzyme extract. The reaction was started by addition of the citrate and was followed at 340nm. It should be noted that cell-free extracts of Aspergillus terreus contain significant levels of alcohol dehydrogenase. In the presence of $NADP^+$, this enzyme is capable of undertaking the oxidation of glycerol, with the resulting formation of NADPH. Since this reaction would interfere with the determination of aconitase activity, a glycerol-free preparation of NADP-dependent isocitrate dehydrogenase was employed.

Isocitrate Dehydrogenase

NADP-dependent isocitrate dehydrogenase was assayed by the method of Kornberg (1955) in which the reduction of $NADP^+$ is followed at 340nm. The 1ml assay mixture contained (final concentrations): 20mM Tris-HCl, pH 8, 10mM $MgCl_2$, 1mM EDTA, 0.2mM $NADP^+$, 4mM disodium DL-isocitrate and enzyme extract. The reaction was

started by addition of the isocitrate.

NAD-dependent isocitrate dehydrogenase was assayed in the same way except that 0.2mM NAD^+ was used instead of $NADP^+$, and 1mM AMP was also included in the assay mixture.

2-Oxoglutarate Dehydrogenase

2-Oxoglutarate dehydrogenase was assayed by the method of Reed et al. (1969) in which the reduction of NAD^+ is followed at 340nm. The 1ml assay mixture contained (final concentrations): 50mM sodium-potassium phosphate buffer, pH 7, 1mM $MgCl_2$, 1mM NAD^+, 0.2mM thiamin pyrophosphate, 0.13mM CoA, 2.6mM cysteine hydrochloride, 2mM disodium 2-oxoglutarate and enzyme extract. The reaction was started by addition of the 2-oxoglutarate.

Assay of pyruvate dehydrogenase was performed in the same way except that 2mM sodium pyruvate was the substrate instead of the 2-oxoglutarate.

Succinate Thiokinase

Succinate thiokinase was assayed by the method of Leitzman et al. (1970) in which the CoA produced in the reaction reduces DTNB to form the yellow thionitrobenzoate anion. The 1ml assay mixture contained (final concentrations): 20mM sodium-potassium phosphate buffer, pH 7.5, 10mM $MgCl_2$, 0.5mM ADP, 0.1mM DTNB, 0.2mM

succinyl-CoA and enzyme extract. The reaction was started by addition of the ADP and followed at 412nm. Succinyl-CoA was prepared using CoA and succinic anhydride by the method of Simon and Shemin (1953).

Succinate Dehydrogenase

Succinate dehydrogenase was assayed by the method of Muller et al. (1968) in which the reduction and concomitant formation of DCPIP is followed at 600nm. The 1ml assay mixture contained (final concentrations): 50mM sodium-potassium phosphate buffer, pH 7.5, 30µM DCPIP, 1mM N-methylphenazinium methosulphate, 2mM KCN, 0.05% Triton X-100, 20mM disodium succinate and enzyme extract. The reaction was started by addition of the succinate.

Fumarase

Fumarase was assayed by the method of Massey (1955) in which the formation of fumarate is followed at 250nm. The 1ml assay mixture contained (final concentrations): 20mM Tris-HCl buffer, pH 8, 10mM $MgCl_2$, 1mM EDTA, 5mM disodium malate and enzyme extract. The reaction was started by addition of the malate.

Malate Dehydrogenase

Malate dehydrogenase was assayed by the method of Ochoa (1958) in which the oxidation of NADH is followed at 340nm. The 1ml assay mixture contained (final concentrations): 100mM Tris-HCl, pH 8, 0.2mM NADH, 0.2mM disodium oxaloacetate and enzyme extract. The reaction was started by addition of the oxaloacetate.

RESULTS AND DISCUSSION

SELECTION OF GROWTH MEDIUM

In the past, Aspergillus terreus (NRRL 1960) has been cultured in a wide variety of growth media. For economic reasons, the industrial production of itaconate by this organism has frequently employed corn-steep liquor as a key ingredient in the culture medium. The organism has also been grown under relatively simple nutritional conditions such as in Czapek-Dox liquid medium. In order to investigate the physiology and biochemistry of itaconate production, previous workers have sought to study the fermentation under strictly defined and, therefore, reproducible conditions. However, not all the media which allow growth also support the production and excretion of itaconate, Czapek-Dox liquid medium being a typical example. Furthermore, the constituency of corn-steep liquor does, by the very nature of its production, vary considerably from batch to batch. In spite of these problems, defined media for the production of itaconate have been described in the literature (Nowakowska-Waszczuk,1973; Rychtera and Wase, 1981; Kautola et al.,1985) as have the effects of iron, copper, calcium and magnesium (summarized by Atkinson and Mavituna, 1983). Ammonium ions appear to be the preferred nitrogen source for itaconate production (Moyer and Coghill, 1945; Nowakowska-Waszczuk, 1973) and the volume, concentration and nature of production of the

inoculum have also been reported to influence the yield of the acid.

Table 1 shows the results of preliminary experiments, performed in this laboratory, aimed at the selection of a high-yielding culture medium. Flasks were inoculated with spores washed from Czapek-Dox agar slopes, as described in the Materials and Methods section, and the media were analysed after a seven-day incubation. Only the medium of Larsen (1957), containing tap-water, was found to be effective in supporting acid production.

In the other cultures where a drop in pH was observed, the amount of titratable acid was negligible. The acid produced in the Larsen medium was shown, by paper chromatography, to have the same Rf value as itaconic acid (Table 2) and appeared as the only acid spot on the chromatogram. Quantitative determination by the spectrophotometric bromination method indicated an itaconate concentration of 30g l^{-1}, thus representing a yield of 30% from the 100g of carbohydrate supplied. A similar medium, but containing distilled water in place of the tap-water, was shown to support growth of the organism, but not itaconate production. Similarly, substitution of potassium nitrate (3g l^{-1}) for the ammonium sulphate in the medium, also prevented acidogenesis. Thus the tap-water medium of Larsen was employed for subsequent studies on itaconate production,

TABLE 1. Acid Production by *Aspergillus terreus* in Different Culture Media.

MEDIUM	INITIAL pH	FINAL pH	TITRATABLE ACID[5] (ml)
Czapek-Dox	7.0	4.0	<1
Czapek-Dox+3g l^{-1} $(NH_4)_2SO_4$	7.0	3.0	1 - 2
Nowakowska-Waszczuk[1]	5.6	3.0	1 - 2
Rychtera[2]	2.8	2.8	1 - 2
Elnaghy[3]	7.0	3.0	1 - 2
Larsen[4]	3.0	1.8	23
Larsen with distilled water	3.0	3.0	1 - 2

Notes:

[1]Nowakowska-Waszczuk (1973). Medium contained (l^{-1} distilled water): glucose, 50g; ammonium sulphate, 3g; $MgSO_4 \cdot 7H_2O$, 0.5g; KH_2PO_4, 0.1g; Fe, 0.1mg; Cu, 0.02mg; Zn, 0.01mg.

[2]Rychtera and Wase (1981). Medium contained (g l^{-1} distilled water): glucose, 50; ammonium sulphate, 3.3; $MgSO_4 \cdot 7H_2O$, 0.8; itaconic acid, 1; KH_2PO_4, 0088; $CuSO_4 \cdot 5H_2O$, 0.004.

[3]Elnaghy and Megalla (1975). Medium contained (g l^{-1} distilled water): glucose, 150; peptone, 10.

[4]Larsen (1957). Constituents given in Materials and Methods.

[5]Volume of 0.1M NaOH required to neutralize a 5ml aliquot of the medium following the fermentation.

TABLE 2. Paper Chromatography of Organic Acid Standards and the Acid Produced by *Aspergillus terreus*. Details are given in Materials and Methods.

ACID	Rf
Citric	0.43
Isocitric	0.52
cis-Aconitic	0.60
Itaconic	0.82
Sample of culture medium	0.82

with nitrate as the nitrogen source in the non-itaconate-producing controls.

There are many possible explanations of why itaconate production was not observed in culture media which had previously been reported to support it. For example, the type and method of inoculation have varied considerably; some authors have employed rich substrates such as nutrient broths or corn-steep liquor at the sporulation stage, a portion of which may have been transferred to the growth medium on inoculation. The methods of subsequent incubation have also differed: surface, shake-flask and fermenter methods have been employed. In addition, the failure of some workers to use highly pure chemicals in the culture media may have added to the confusion. Finally, it is conceivable that Aspergillus terreus (NRRL 1960) has changed physiologically over the years, by natural mutation, and that different laboratories have unknowingly been using different varieties of this organism. Clearly, the tap-water in the medium of Larsen contained the balance of key ions and/or trace elements which was essential for the production of itaconate by the strain of Aspergillus terreus employed in the present study.

CITRIC ACID CYCLE ACTIVITY

The fundamental importance of the citric acid cycle in primary metabolism, and its significance to the microorganism cultured in a minimal growth medium, has been detailed in the Introduction. The occurrence of the enzymes of the citric acid cycle in fungi has been reviewed by Niederpruem (1965) and more recent data is available for Neurospora (Flavell and Fincham, 1968), Saccharomyces (Gosling and Duggen, 1971) and Blastocladiella (Khouw and McCurdy, 1969). Moreover, citric acid cycle enzyme activities have also been detected in Aspergillus nidulans (McCullough et al., 1977) and Aspergillus niger (Ng et al., 1973; Kubicek and Rohr, 1977; Mattey, 1977; Szczodrak, 1981). Consequently, the reported inability of Nowakowska-Waszczuk (1973) to detect any citric acid cycle activity in mitochondria isolated from Aspergillus terreus (NRRL 1960) prompted an investigation, in this laboratory, into the activities of the individual enzymes of the cycle. This study was considered especially prudent because of the significance of the citric acid cycle to the proposed pathway for the production of itaconate (via cis-aconitate decarboxylation) in this organism.

Aspergillus terreus was cultured under itaconate-producing and non-itaconate-producing conditions (as described in the previous section). After 100-hours

growth, cell-free extracts were made and assayed directly for citric acid cycle enzymes. The results are shown in Table 3. Citrate synthase, aconitase, NADP-dependent isocitrate dehydrogenase, succinate dehydrogenase, fumarase and malate dehydrogenase were readily detected. NAD-dependent isocitrate dehydrogenase activity, regarded to occur exclusively in the mitochondrion, was not detected. This enzyme has been shown to be present in Aspergillus niger (Mattey, 1977) but with one hundredth of the activity of its NADP-dependent counterpart. However, McCullough et al. (1977) have reported its apparent absence from Aspergillus nidulans. It is possible, therefore, that the enzyme does exist in Aspergillus terreus and Aspergillus nidulans, but with very low activity, or in a labile state which becomes inactive on extraction.

2-oxoglutarate dehydrogenase activity was not detected in any of the three extractions shown in Table 3, but repeats of these experiments (results not shown) did indicate the presence of slight activity (less than 1nmole min^{-1} mg^{-1}). McCullough et al. (1977) also failed to confirm the presence of this enzyme in sucrose-grown Aspergillus nidulans, but significant activity was present in acetate-grown mycelia. In 1977, Kubicek and Rohr reported the absence of this enzyme in Aspergillus niger, but, more recently (Meixner-Monori et al., 1985) have been able to detect activity in extracts in which a

TABLE 3. Comparison of the Specific Activities of Citric Acid Cycle Enzymes in Itaconate-producing (I) and non-itaconate-producing (II) Mycelia of ASPERGILLUS TERREUS.

Three different extraction buffers were employed, as described in Materials and Methods, Phosphate (P), HEPES (H) and Tris (T).

	BUFFER	ENZYME ACTIVITY (nmol/min/mg)	
		I	II
CITRATE SYNTHASE	P	300	240
	H	290	260
	T	220	290
ACONITASE	P	3.4	8.5
	H	28	12
	T	6.7	4.5
NADP-ISOCITRATE DEHYDROGENASE	P	110	86
	H	76	79
	T	58	37
NAD-ISOCITRATE DEHYDROGENASE	P	-	-
	H	-	-
	T	-	-
2-OXOGLUTARATE DEHYDROGENASE	P	-	-
	H	-	-
	T	-	-
SUCCINATE THIOKINASE	P	-	-
	H	-	-
	T	2.2	3.7
SUCCINATE DEHYDROGENASE	P	-	-
	H	3.5	1.8
	T	3.6	4.2
FUMARASE	P	100	190
	H	200	150
	T	38	35
MALATE DEHYDROGENASE	P	4800	2700
	H	4900	4900
	T	5400	5500

high protein concentration was maintained. It is therefore possible that 2-oxoglutarate dehydrogenase is present in all Aspergillus spp., but with a reduced activity when a carbohydrate carbon source is used in the culture medium (in which case, the citric acid cycle functions in a primarily biosynthetic mode, and energy is produced by glycolysis). Failure to detect activity of this enzyme in cell-free extracts may be due to its relative instability in dilute solution; this problem might be alleviated to some extent by maintaining a high protein concentration throughout the extraction procedure. Efforts were made to elevate the activity of this enzyme in Aspergillus terreus by attempting to culture the organism in media in which acetate, citrate, succinate and glutamate were the respective sole sources of carbon. However, no growth was obtained under these conditions.

There are no reports in the literature of attempts to measure succinate thiokinase activity in Aspergillus spp. The results presented in Table 3 show that the enzyme is present in Aspergillus terreus, but with relatively low activity. The enzyme was shown to be specific for ADP as the nucleotide substrate. No GDP-dependent activity was found.

It may therefore be concluded that, contrary to the findings of Nowakowska-Waszczuk (1973), Aspergillus

terreus (NRRL 1960) possesses many citric acid cycle enzymes. A comparison of the levels of the enzymes found in Aspergillus terreus with those found by other authors in Aspergillus nidulans and Aspergillus niger is given in Table 4. It can be seen that the relative activities of the individual enzymes are quite similar and may be proposed as being typical of those present in Aspergillus spp. generally.

The comparison of the respective enzyme levels in the itaconate-producing mode with those in the non-itaconate-producing mode (Tables 3 and 4) indicated no obvious differences. Thus, there was no apparent gross change in the activity of the citric acid cycle associated with the production of itaconate. Recently, however, Winskill (1983) has detected an eight-fold difference in NADP-dependent isocitrate dehydrogenase activity between the growth and acid-production phases of an itaconate-producing strain of Aspergillus terreus (M490). This drop in isocitrate dehydrogenase activity was accompanied by a doubling of citrate synthase activity. Winskill (1983) therefore proposed that the production and excretion of itaconate by Aspergillus terreus is facilitated by a reduction in the activity of isocitrate dehydrogenase. Nevertheless, whether this reduction causes, or is caused by, itaconate production has yet to be resolved. This issue could be clarified by monitoring acidogenesis and isocitrate dehydrogenase activity more

TABLE 4. Comparison of the Levels of Citric Acid Cycle Enzymes in three Aspergillus spp.

	SPECIFIC ACTIVITY (nmol. min^{-1} mg^{-1})			
	terreus		nidulans+	niger*
	I	II		
Citrate Synthase	270	290	650	250
Aconitase	13	19	73	75
NADP-Isocitrate Dehydrogenase	81	67	150	25
NAD-Isocitrate Dehydrogenase	0	0	0	2
2-Oxoglutarate Dehydrogenase	<1	<1	0	0
Succinate Thiokinase	1	1	N/D	N/D
Succinate Dehydrogenase	2	2	16	2
Fumarase	112	125	655	250
Malate	5000	4300	9900	3000

I = Itaconate-producing

II = Non-itaconate-producing

+From McCullough et al., 1977
*From Kubicek and Rohr, 1977

closely throughout the time course of a fermentation. If the onset of itaconate production were shown to precede the fall in enzyme activity, the latter occurrence could be taken to be of secondary importance. If the proposal of Winskill (1983) also applied to Aspergillus terreus (NRRL 1960), the activity of the NADP-dependent isocitrate dehydrogenase observed in the itaconate-producing mode should have been significantly lower than in the non-itaconate-producing counterpart. Clearly, this was not the case. Moreover, results to be presented in a later section cast further doubt on the importance of this enzyme in the production of itaconate by Aspergillus terreus (NRRL 1960).

ENZYME-LINKED ASSAY FOR ITACONATE

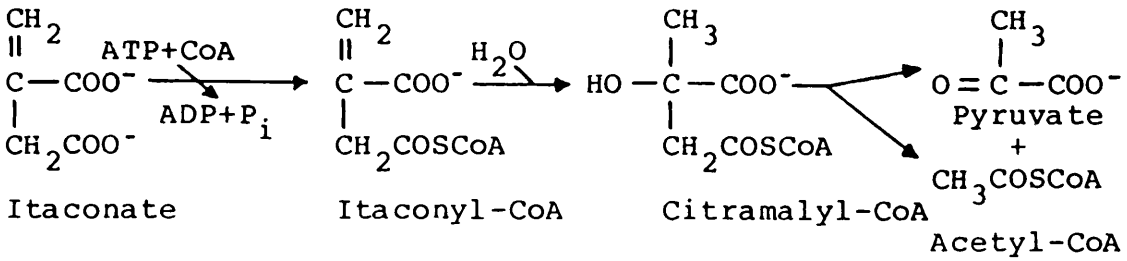
The confirmation of the presence of the necessary citric acid cycle enzymes in Aspergillus terreus during itaconate production added further support to the theory that itaconate biosynthesis occurs via the decarboxylation of cis-aconitate. However, the manometric method for the assay of CAD, originally employed by Bentley and Thiessen (1957), was considered to be an inconvenient procedure for more detailed studies of the enzyme. Furthermore, the previously published assays for itaconate were not suitable for enzymological studies. The iodometric titration method of Friedkin (1945), although accurate and sensitive, is very time-consuming and involves many different steps. The adaptation of this method to spectrophotometry by Adler et al. (1957) resulted in a more rapid assay procedure, but at the expense of a loss in accuracy. In addition, the 'Bromine Reagent' used in these two assays may also react with other unsaturated compounds present in the sample. Different types of chromatographic detection techniques have also been reported, i.e. gas-liquid chromatography (Tabuchi et al., 1975) and isotachophoresis (Horitzu et al.,1983). However, these methods also require pre-treatment of the sample.

A further disadvantage of the above techniques for the determination of itaconate is that they all have to be used in a discontinuous mode. For enzymological studies,

the ideal assay system should also be amenable to usage in a continuous mode; it should,therefore, operate effectively under physiological conditions and cause minimal interference to the system under study. Consequently, in the absence of a readily exploitable physical or chemical property of itaconate, an enzyme-linked detection system was sought. The investigations which led to the development of such an assay are described below.

Enzymes for which Itaconate is a Substrate

Three different types of enzyme systems which are capable of using itaconate as a substrate have been described (Bentley and Thiessen, 1962). An ultraviolet-induced mutant of Aspergillus terreus has been shown to contain itaconate oxidase, which, in the presence of molecular oxygen, converts itaconic acid to itatartaric acid (Arpai, 1959). Wang et al. (1961) demonstrated the presence of an enzyme system in the mitochondria of rat and guinea pig liver which catalyses the conversion of itaconate to pyruvate and acetyl-CoA. Finally, Cooper and Kornberg (1964) showed that pyruvate and acetyl-CoA were also the end-products of itaconate dissimilation in itaconate-grown Pseudomonas spp.; these authors then demonstrated the route of catabolism to be:



This had also been shown to be the route of itaconate breakdown in liver mitochondria. In both the mitochondrial and microbial systems, the enzyme which catalyses the formation of itaconyl-CoA from itaconate, ATP and CoA, appears to be succinate thiokinase. Itaconyl-CoA hydratase and citramalyl-CoA lyase perform the two successive steps respectively. However, the two enzyme systems are not completely identical, the major difference being that Pseudomonas spp. possess an additional enzyme for the activation of itaconate, itaconate - CoA transferase, which is capable of catalysing the following conversions:

itaconate + succinyl-CoA ⟩(itaconyl-CoA + succinate

and

citramalate + succinyl-CoA ⟩(citramalyl-CoA + succinate

This enzyme is not present in the mitochondrial system.

Thus, in the presence of a cell-free extract of itaconate-grown Pseudomonas sp., ATP, CoA and Mg^{2+}, stoichiometric conversion of itaconate to pyruvate and acetyl-CoA is obtained. During their investigations, Cooper and Kornberg (1964) used two methods to monitor the formation of pyruvate from itaconate continuously. In the first, NADH and lactate dehydrogenase were added to the assay system at pH 8. Pyruvate formation from itaconate was thus followed spectrophotometrically at 340nm by virtue of the decrease in absorbance which accompanies NADH oxidation. In the second method,

phenylhydrazine was added to the assay system at pH 6.8, and the increase in absorbance at 315nm, concomitant with the formation of pyruvate phenylhydrazone, was monitored.

Itaconate Utilization by Pseudomonas aeruginosa (PA01)

Cooper and Kornberg (1964) had studied the catabolism of itaconate in Pseudomonas B_2aba. However, because of the close similarity between the pathway elucidated by these authors and that which had been shown to be active in mammalian liver mitochondria, it was considered reasonable to suppose that the same pathway existed in other species of Pseudomonas which are capable of growth on itaconate. Therefore, in preliminary experiments, performed in this laboratory, a wild-type strain of Pseudomonas aeruginosa (PA01) was cultured in a minimal salts medium containing itaconate as the sole source of carbon (details are given in Materials and Methods). Following growth, harvesting, and extraction of the cells, the resulting unfractionated enzyme extract was analysed for its ability to catalyse the conversion of itaconate to pyruvate.

To a 1ml cuvette were added: 880µl of sodium-potassium phosphate buffer, pH 7.5, 20µl of 10mM CoA, 20µl of 100mM ATP, 20µl of 100mM $MgCl_2$, 10µl of enzyme extract (approximately 100µg of soluble protein), 20µl of 10mM NADH, 10µl of rabbit muscle lactate dehydrogenase (0.5 units) and 20µl of 100mM disodium itaconate. The reaction

was followed at 340nm in a dual-beam spectrophotometer at 25°C, without a reference cuvette, and was initiated by addition of the itaconate. Following a 2-3min period of hysteresis, a linear rate of decrease in absorbance was observed. The rate was shown to be directly proportional to the volume of extract added. No activity was detected if any of the listed constituents had been omitted from the cuvette. However, if 20µl of 10mM succinyl-CoA (preparation of this is described on page 33) was also included in the assay mixture, the requirement for ATP and CoA disappeared and the activity of the system doubled. This was taken as evidence for the presence of itaconate - CoA transferase in the enzyme extract. The close agreement between the above results and those obtained by Cooper and Kornberg (1964) thus confirmed the similarity between itaconate catabolism in the two organisms Pseudomonas B_2aba and Pseudomonas aeruginosa (PA01).

Optimization of Conditions for Itaconate Assay

In the experiments described above, a linear rate of decrease in absorbance was also observed when the cuvette contained only buffer, NADH and enzyme extract. The rate of this decrease was considerably smaller than the rates of itaconate consumption but nevertheless interfered with the determination of the latter. The activity of this 'NADH oxidase' was abolished in the presence of 20mM sodium cyanide. However, at this concentration, sodium

cyanide had the effect of increasing the pH of the assay system to a value of about 9. At such a high pH, magnesium phosphate salts precipitated out of solution and thus interfered with the absorbance determinations. In addition, the pH optimum of CAD had been shown to be less than 6 by Bentley and Thiessen (1957) and any proposed linking system for this enzyme would need to be capable of operating around this pH. Consequently, the suitability of using phenylhydrazine for the detection of pyruvate was investigated.

Figure 1 shows the increase in absorbance caused by the reaction of various amounts of pyruvate with phenylhydrazine; the latter compound was present in excess. Reaction mixtures contained 100µl of 100mM phenylhydrazine, 20-200µl of 1mM sodium pyruvate and, to a total volume of 1ml, 20mM sodium-potassium phosphate buffer, pH 7.0. After 30min at 25°C, the change in absorbance at 315nm was measured against a blank which contained no pyruvate. From Fig. 1, the absorption coefficient for pyruvate phenylhydrazone was calculated to be 1.4×10^4 l mol^{-1} cm^{-1}.

Replacing pyruvate in the reaction mixture with itaconate and the itaconate-consuming system led to the results shown in Fig. 2. The 1ml reaction mixtures contained 100µl of 100mM phenylhydrazine, 20µl of 100mM $MgCl_2$, 20µl of 100mM ATP, 20µl of 10mM CoA, 20-100µl of 1mM disodium

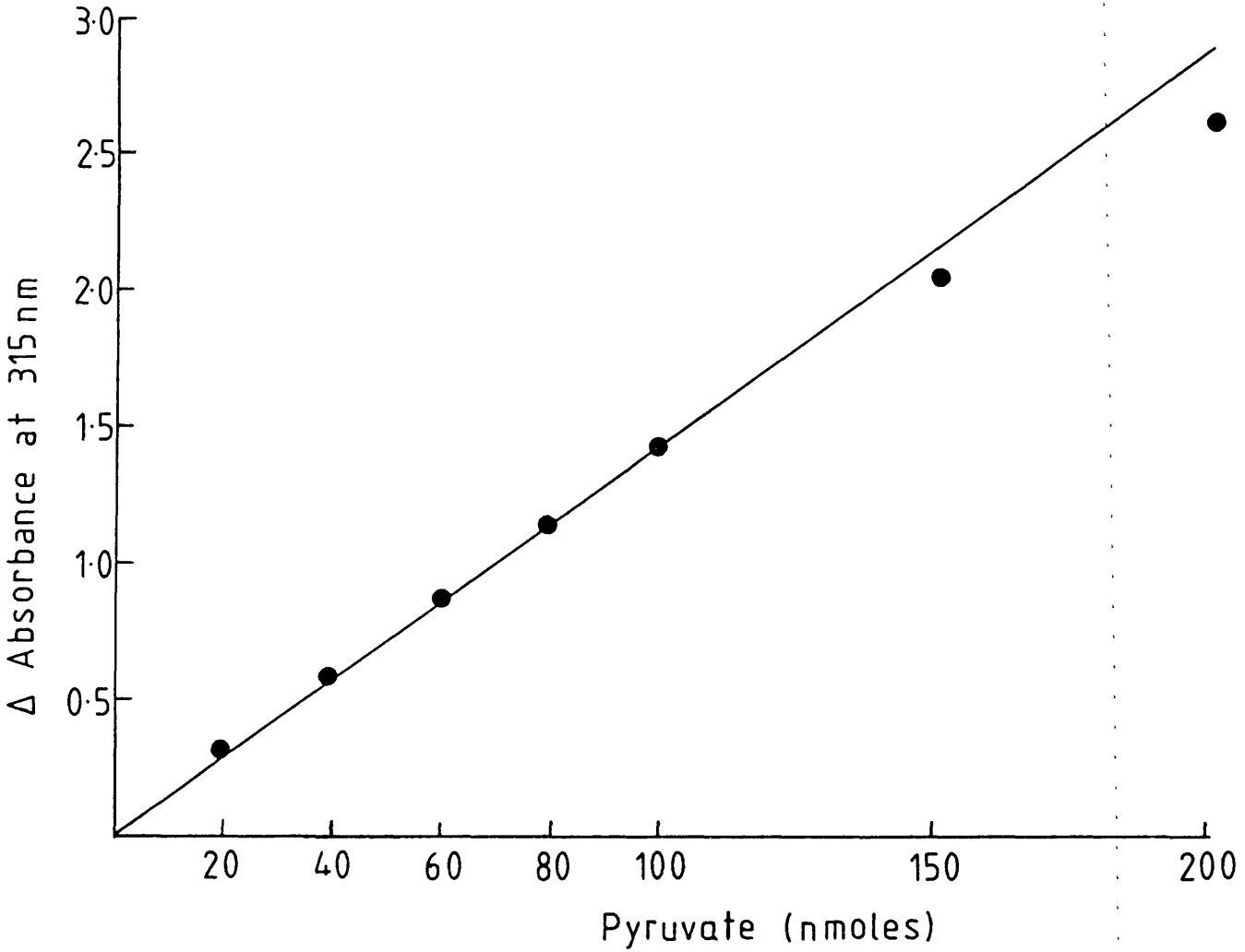


FIG. 1. Change in absorbance at 315nm caused by the reaction of pyruvate with excess phenylhydrazine.

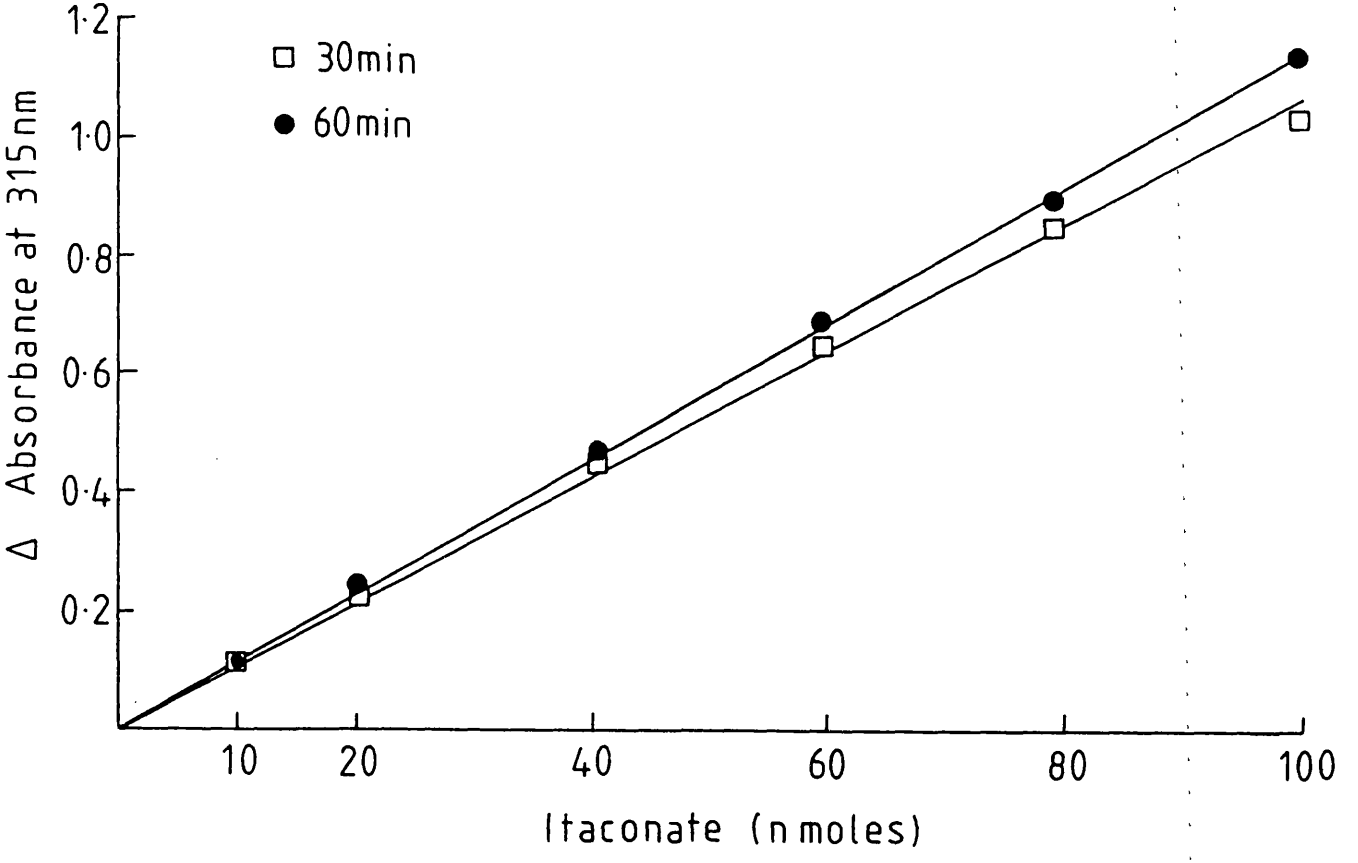


FIG. 2. Pyruvate phenylhydrazone formation from itaconate in the presence of excess phenylhydrazine and the itaconate-consuming system. The results of two incubations are shown.

itaconate, 50µl of enzyme extract (approximately 1mg of soluble protein) and, to a total volume of 1ml, 20mM sodium-potassium phosphate buffer, pH 7.0. After 30min at 25°C, the change in absorbance at 315nm was measured against a blank which contained no itaconate. The absorbances were then redetermined following a further 30min incubation period. Clearly, the standard curve obtained (after the 60min incubation) is suitable for the determination of itaconate. The assay was effective over the range 20-100nmoles of itaconate.

The Accuracy of the Enzyme-Linked Assay for Itaconate

Although at the lower concentrations of itaconate the change in absorbance due to the formation of pyruvate phenylhydrazone (Fig. 2) is in close agreement with those values obtained for the pyruvate standard (Fig. 1), at the higher concentrations of itaconate the two sets of values differ significantly. Thus, the change in absorbance cased by 100nmoles of itaconate was 1.13, whereas the change caused by the same amount of pyruvate was 1.40. Such discrepancies possibly arose from the low solubility of pyruvate phenylhydrazone in the enzyme-linked assay system, or the diminished accuracy of the spectrophotometer at the higher absorbance values. The following experiment was therefore performed in order to confirm the stoichiometric conversion of itaconate to pyruvate by the Pseudomonas extract.

A standard curve for the determination of 10-100nmoles of pyruvate phenylhydrazone was constructed under the conditions of the itaconate assay: the 1ml reaction mixtures contained 100µl of 100mM phenylhydrazine, 20µl of 100mM $MgCl_2$, 20µl of 100mM ATP, 20µl of 10mM CoA, 10-100µl of 1mM sodium pyruvate, 100µl of enzyme extract (approximately 2mg of soluble protein) and, to a total volume of 1ml, 20mM sodium-potassium phosphate buffer, pH 7.0. The change in absorbance at 315nm was then determined following 60min incubation at 25°C. The blank, against which the above values were measured, contained no sodium pyruvate. The standard curve thus obtained is shown in Fig 3. The experiment was then repeated with 1mM disodium itaconate in place of the sodium pyruvate. These results are also presented in Fig. 3. The changes in absorbance caused by the known concentrations of itaconate were then read from the pyruvate standard curve to give corresponding concentrations of pyruvate. The values of pyruvate concentration thus obtained have been plotted against itaconate concentration in Fig. 4, and the stoichiometric conversion of itaconate to pyruvate is therefore confirmed.

Standard Conditions for the Determination of Itaconate

All subsequent experiments which are to be described in this report up to p.72, involving the detection or the determination of itaconate, consisted of a cuvette which

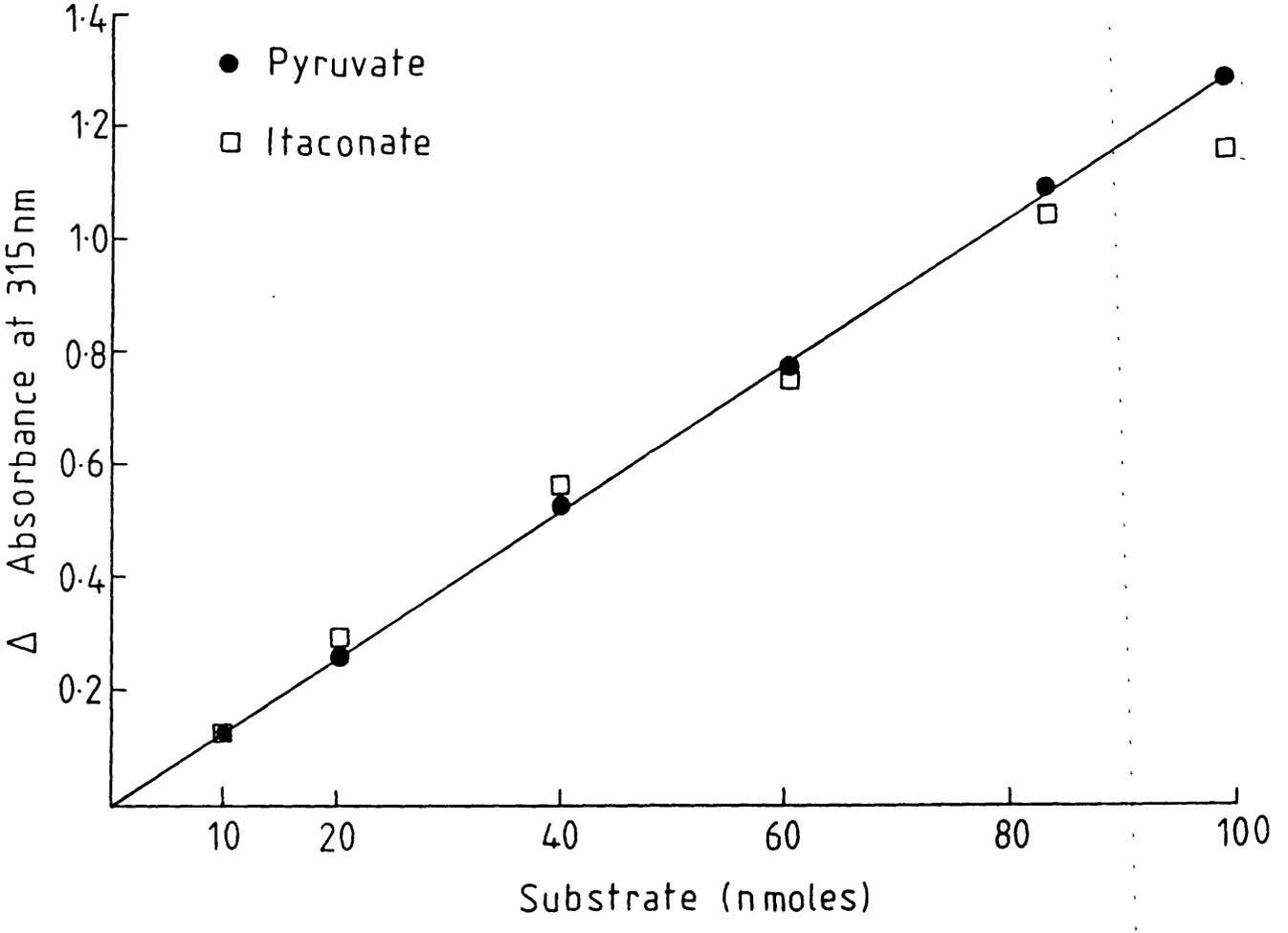


FIG. 3. Pyruvate phenylhydrazone formation from pyruvate (●) and itaconate (□) in the presence of phenylhydrazine and the itaconate-consuming system in both cases.

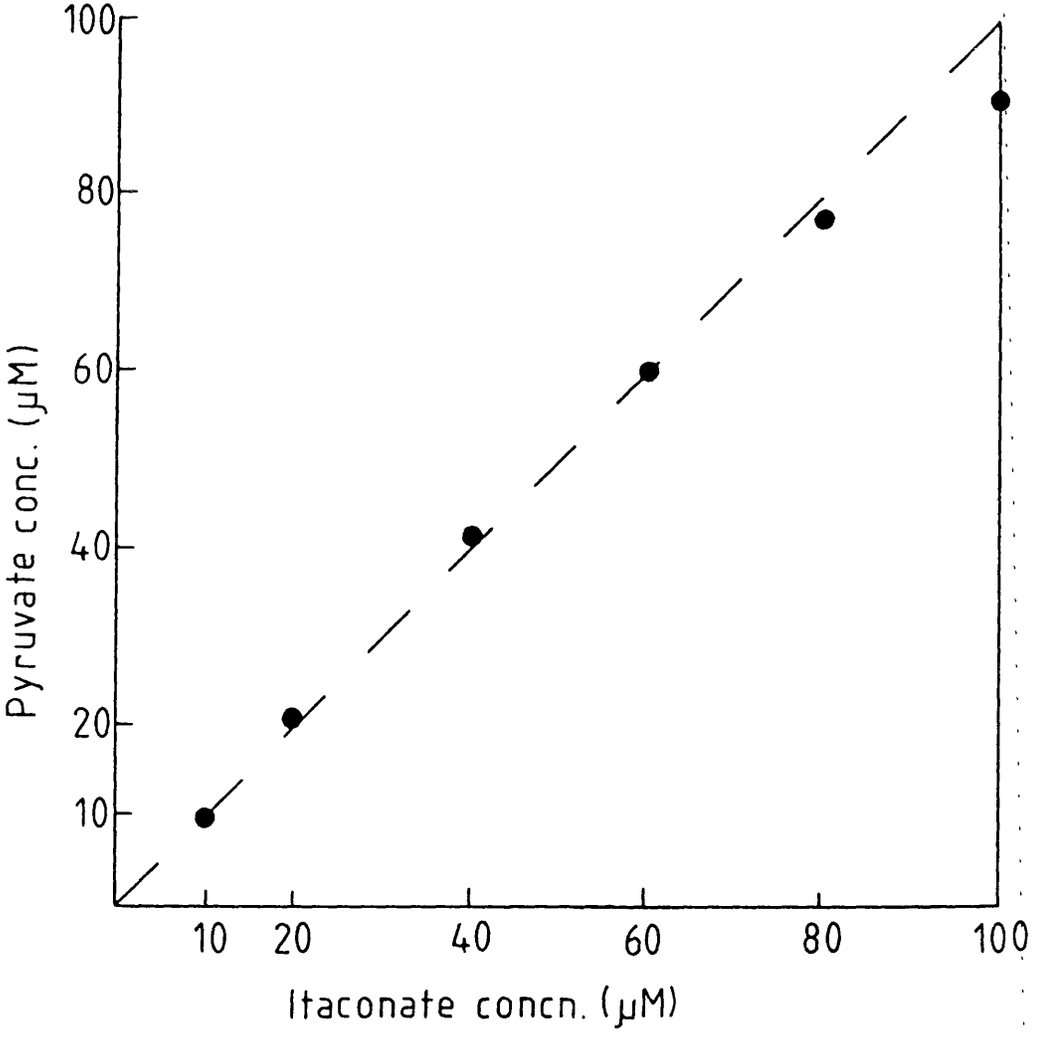


FIG. 4. Demonstration of the stoichiometric conversion of itaconate to pyruvate. Details are given in the text.

contained (unless otherwise stated) in a total volume of 1ml: 20mM sodium-potassium phosphate buffer, pH 7.0, 10mM phenylhydrazine hydrochloride, 2mM $MgCl_2$, 2mM ATP, 0.2mM CoA and Pseudomonas extract (amount as indicated). The details of all other additions will be indicated where necessary. The reactions were initiated by the addition of the itaconate-containing sample.

Determination of the Km of the Itaconate-Consuming System

Itaconate concentrations over the range 10µM to 10mM were included in the standard itaconate assay system which contained 10µl of the enzyme extract (approximately 200µg of soluble protein). Initial reaction velocities were then determined and the results are presented in Fig. 5 in the form of a double reciprocal plot. From this, the Km value of the enzyme-linked system for itaconate was determined to be 0.30mM. This value was confirmed by analysis of the data using the computer-fitted direct linear plot method of Eisenthal and Cornish-Bowden (1974).

Significantly, the itaconate concentrations for which the itaconate assay is effective (10 to 100µM) are notably less than the Km value of the system for itaconate (300µM). It is unfortunate, therefore, that larger amounts of Pseudomonas extract have to be included in the itaconate assay system than would be the case with a lower Km value. However, the relatively high Km value

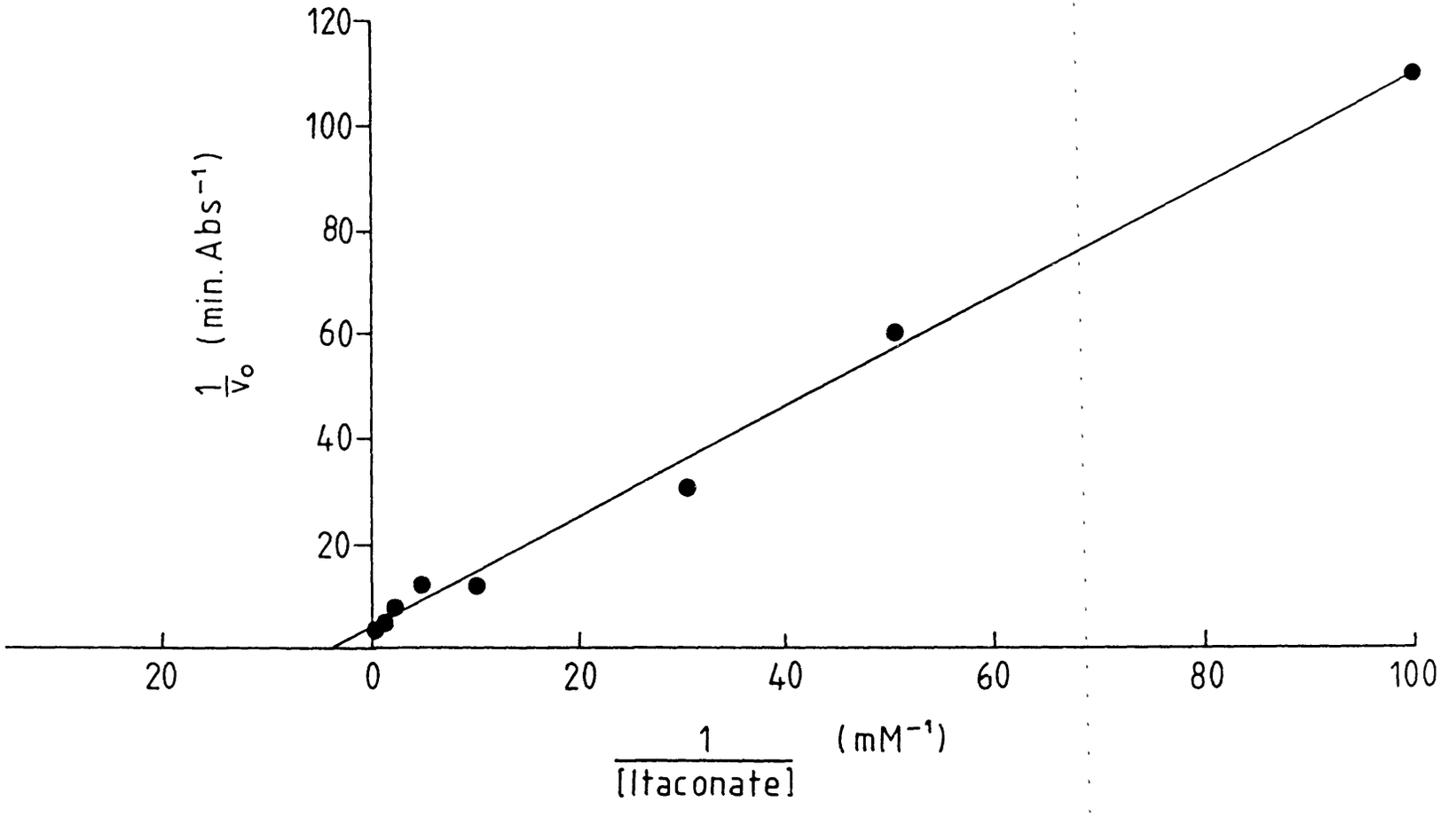


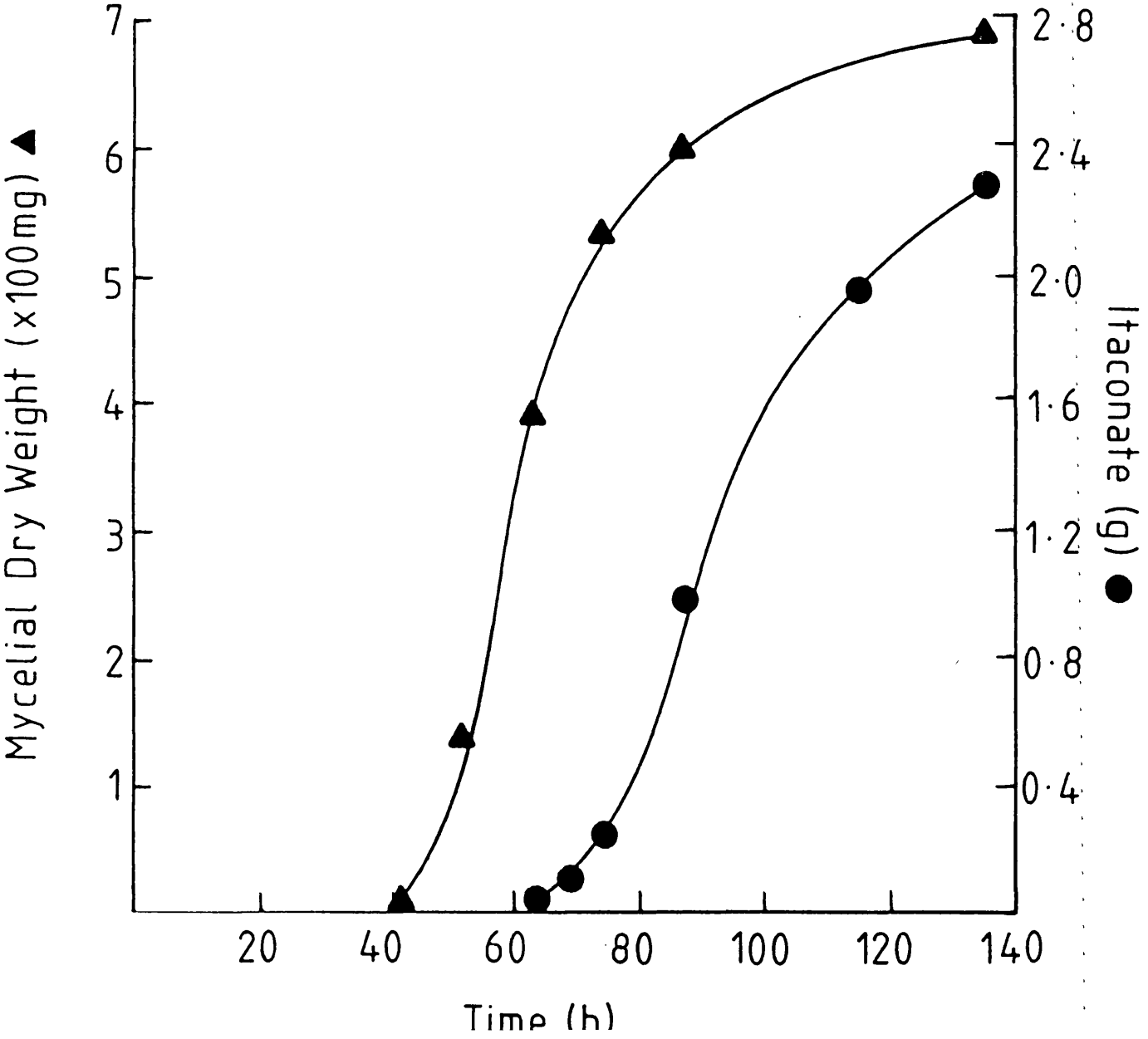
FIG. 5. Double-reciprocal plot for the determination of the Km of the itaconate assay system for itaconate.

probably reflects the fact that the activation of itaconate is catalysed by an enzyme which appears to have been sequestered for this role (succinate thiokinase) and for which itaconate is not, primarily, a natural substrate.

Suitability of the Assay for the Monitoring of Itaconate Production during a Fermentation of Aspergillus terreus. Aspergillus terreus was cultured in replicate flasks under conditions of itaconate production. At selected times, the mycelium was harvested for growth determination and the culture medium was analysed for itaconate. Samples of culture medium, suitably diluted with 20mM sodium-potassium phosphate buffer, pH 7.0, were added to the standard itaconate-consuming system (100µl of sample, 100µl of Pseudomonas extract) and assayed for itaconate against a blank which contained no culture medium. The results are shown in Fig. 6. The time-course of itaconate production in relation to growth is in close agreement with those presented by previous authors (e.g. Winskill, 1983), and the concentrations of itaconate determined by this method were shown to be identical with those values obtained for duplicate samples assayed for itaconate by the method of Adler et al. (1957).

The enzyme-linked assay for itaconate developed here is thus suitable for following itaconate production

FIG. 6. Suitability of the enzyme-linked assay to monitor itaconic acid production during fermentation of ASPERGILLUS TERREUS.



throughout the time-course of a fermentation of *Aspergillus terreus*. None of the other constituents of the medium appeared to interfere with the assay system.

SUITABILITY OF *PSEUDOMONAS AERUGINOSA* PA01 EXTRACTS TO THE DETECTION OF CAD IN *ASPERGILLUS TERREUS*

As indicated previously, the primary advantage of an enzyme-linked assay for itaconate should be its suitability for the continuous monitoring of itaconate production in cell-free extracts of *Aspergillus terreus*, and associated enzymological studies. It should be possible to use the standard itaconate-consuming system and, with the addition of *cis*-aconitate and an extract of itaconate-producing *Aspergillus terreus* (assumed to contain CAD activity), follow itaconate formation spectrophotometrically. The following sections describe the difficulties initially encountered in attempting such an adaptation of the assay.

Aconitase and Isocitrate Lyase in the Pseudomonas *Extract*

Addition of 50µl of 100mM disodium *cis*-aconitate to the standard itaconate assay system (containing 20µl of *Pseudomonas* extract) resulted in a rapid linear rate of increase in absorbance at 315nm after a brief (approximately 1min) lag period. The initial velocity was shown to be directly proportional to the amount of *Pseudomonas* extract added, and identical with that obtained if 50µl of 100mM disodium DL-isocitrate was substituted for the *cis*-aconitate. However, when isocitrate was the reaction initiator, no lag period was observed before the development of a linear rate. This

suggested that the observed increase in absorbance was due to the formation of glyoxylate phenylhydrazone, produced by the action of isocitrate lyase. When cis-aconitate was the reaction initiator, aconitase will have facilitated the formation of isocitrate which could then be converted to glyoxylate and succinate under the action of isocitrate lyase. These responses were also obtained in the absence of CoA and ATP, which are not required for aconitase or isocitrate lyase activity.

The rate of formation of glyoxylate phenylhydrazone from cis-aconitate was shown to take place at three times the rate at which pyruvate phenylhydrazone was formed from an identical concentration of itaconate. Clearly, this prevented the detection of any putative CAD activity in extracts of Aspergillus.

The Effects of Succinate on the Pseudomonas Extract

Succinate, a product of the isocitrate lyase reaction, also reduces the activity of the enzyme by product inhibition (McFadden, 1969). Furthermore, succinate is also an activator of the itaconate-consuming system (see p.48) because, in the complete system, in vitro formation of succinyl-CoA occurs from succinate, ATP and CoA through the action of succinate thiokinase. The succinyl-CoA so formed then reacts with itaconate to form itaconyl-CoA and succinate in a reaction catalysed by itaconate - CoA transferase. This method of itaconyl-CoA

formation is more rapid than that of straightforward itaconate activation by succinate thiokinase. Thus, in experiments performed with the standard itaconate assay system (containing 20µl of Pseudomonas extract), in the presence of 10mM disodium succinate, 50% inhibition of the rate of glyoxylate phenylhydrazone formation from cis-aconitate was observed as well as 50% activation of the rate of pyruvate phenylhydrazone formation from itaconate. The optimum conditions under which to attempt the assay of CAD, therefore appeared to favour the presence of succinate.

The Effects of Different Buffers on the Pseudomonas Extract

In order to attempt further inhibition of the isocitrate lyase and activation of the itaconate-consuming reactions, the standard conditions for the itaconate assay were altered. Replacing the 20mM sodium-potassium phosphate buffer, pH 7.0, normally used, with 100mM sodium succinate buffer, pH 6.5, led to almost 100% inhibition of the isocitrate lyase reaction; unfortunately, however, a similar inhibition of the itaconate-consuming reactions was also brought about. Sodium-potassium phosphate buffer, pH 7.0, at a concentration of 100mM, also produced almost complete inhibition of the conversion of itaconate to pyruvate. It thus appears that the itaconate-consuming system functions best in conditions of low ionic strength.

Bentley and Thiessen (1957) assayed CAD in 200mM phosphate buffer, pH 5.6. However, attempts to detect pyruvate formation from itaconate in the Pseudomonas system operating in 20mM sodium-potassium phosphate buffer, pH 5.6, proved negative.

In summary, these experiments demonstrated that the enzymes catalysing the conversion of itaconate to pyruvate in the Pseudomonas extract were more active in conditions of low ionic strength and near-neutral pH. Moreover, the system was more active in the presence of succinate which had the effect of inhibiting isocitrate lyase, an enzyme catalysing a reaction which interfered with the itaconate assay when cis-aconitate was also present.

Detection of CAD Activity in Aspergillus terreus

Following the findings of the previous experiments, in which the optimum conditions for the detection of itaconate and the simultaneous inhibition of isocitrate lyase had been determined, the experiments described below were performed in an effort to detect CAD activity in itaconate-producing Aspergillus terreus

The mycelium from a four-day old culture of itaconate-producing Aspergillus terreus was harvested, and a cell-free extract was prepared in 20mM succinate buffer, pH 6.5. To 4ml of this extract was added 1ml of

100mM disodium cis-aconitate. A 120µl aliquot was removed immediately after mixing (zero time) and was transferred to a pre-heated test-tube in a boiling water-bath for 3min. The precipitated protein was removed by centrifugation in a microfuge. The remainder of the cis-aconitate/ Aspergillus extract mixture was incubated at 30°C. After 10min, another 120µl aliquot was withdrawn and treated in an identical manner to the previous one.

A 50µl sample of the deproteinized zero-time aliquot was added to a standard itaconate assay mixture (with 40µl of Pseudomonas extract) which contained 20mM succinate buffer, pH 6.5, instead of the usual phosphate buffer. A further 50µl sample was added to a duplicate assay mixture from which the CoA and ATP had been omitted. The absorbance of the former mixture was then monitored at 315nm in a dual-beam spectrophotometer with the latter mixture acting as the blank. In this way, the rate of formation of glyoxylate phenylhydrazone from the cis-aconitate present in the sample would be the same in each cuvette, and only the ATP- and CoA-dependent formation of pyruvate phenylhydrazone from itaconate would be detected.

No change in absorbance was observed for the zero-time sample, thus suggesting the absence of itaconate. However, when the 10min sample was analysed in the same way, a linear rate of increase in absorbance was detected

which followed a short (5min) time lag. This suggested that itaconate formation had occurred in the mixture of Aspergillus extract and cis-aconitate during the 10min incubation. Furthermore, it was shown that if the Aspergillus extract had been heated to 90°C for 5min and then cooled, prior to incubation with cis-aconitate, no itaconate formation occurred, even after prolonged incubations.

These results indicate the occurrence of enzymic conversion of cis-aconitate to itaconate in a cell-free extract of itaconate-producing Aspergillus terreus. However, even in 20mM succinate buffer, accurate quantitation of the itaconate formed was not possible because of the isocitrate lyase side-reaction. The itaconate-consuming reaction could not be followed to completion before the absorbance of the cuvettes became too high due to glyoxylate phenylhydrazone accumulation; in other words, the ratio of isocitrate lyase activity to itaconate-consuming activity was too high.

Cooper and Kornberg (1964) had shown that all of the enzymes involved in the conversion of itaconate to pyruvate and acetyl-CoA precipitated out of solution at between 30% and 60% ammonium sulphate saturation of the Pseudomonas extract. An attempt was therefore made to repeat this fractionation procedure, since it was conceivable that either the aconitase or isocitrate lyase

activities could be removed in this way. The results of a 40% to 60% ammonium sulphate fractionation experiment are presented in Table 5. The activities of the conversions itaconate to pyruvate, cis-aconitate to glyoxylate and isocitrate to glyoxylate were determined under the standard conditions of the itaconate assay (with 5mM cis-aconitate and 5mM isocitrate substituting for the 5mM itaconate in the last two assays, respectively). The conversion of cis-aconitate to isocitrate was determined by linking the reaction to isocitrate dehydrogenase as previously described for the assay of aconitase. Although the ratio of the specific activities of cis-aconitate-to-glyoxylate : itaconate-to-pyruvate was reduced from 3 : 1 to 1.5 : 1, a far more substantial reduction was considered necessary.

TABLE 5. Partial Purification of the PSEUDOMONAS extract

		VOLUME (ml)	PROTEIN (mg/ml)	SPECIFIC ACTIVITY (units/mg)	TOTAL UNITS	YIELD (%)	PURITY
Itaconate→Pyruvate	'Crude'	8.5	30	0.0046	1.2	100	1
	Protamine Sulphate	6.0	15	0.012	1.1	92	2.6
	40% Ammon. Sulphate Supernatant	5.0	10	0.0084	0.42	35	1.8
	60% Ammon. Sulphate Pellet	1.0	20	0.013	0.26	22	2.8
Aconitate→Glyoxylate	'Crude'	8.5	30	0.012	3.1	100	1
	Protamine Sulphate	6.0	15	0.017	1.5	49	1.4
	40% Ammon. Sulphate Supernatant	5.0	10	0.019	0.95	31	1.6
	60% Ammon. Sulphate Pellet	1.0	20	0.019	0.38	12	1.6
Aconitate→Isocitrate	'Crude'	8.5	30	0.54	138	100	1
	Protamine Sulphate	6.0	15	0.55	50	36	1.0
	40% Ammon. Sulphate Supernatant	5.0	10	0.60	30	22	1.1
	60% Ammon. Sulphate Pellet	1.0	20	0.48	10	7	0.9
Glyoxylate→Isocitrate	'Crude'	8.5	30	0.014	3.6	100	1
	Protamine Sulphate	6.0	15	0.016	1.5	41	1.1
	40% Ammon. Sulphate Supernatant	5.0	10	0.025	1.2	33	1.8
	60% Ammon. Sulphate Pellet	1.0	20	0.084	1.7	47	6.0

CONTINUOUS ENZYME-LINKED ASSAY FOR CAD USING *PSEUDOMONAS AERUGINOSA* (PAC 501)

Skinner and Clarke (1968) isolated an isocitrate lyase-deficient mutant of wild-type *Pseudomonas aeruginosa* 8602 following mutagenesis with ethylmethane sulphonate. (In the publication of this work, the authors referred to the mutant as *Pseudomonas aeruginosa* At 1; however the organism has since been renamed *Pseudomonas aeruginosa* PAC 501.) The mutant was selected on the basis of its inability to grow in media in which acetate was the sole source of carbon, and, when the carbon source for growth was propionate or succinate, only trace amounts of isocitrate lyase were detected.

The kind donation of a culture of *Pseudomonas aeruginosa* PAC 501 by Prof. Clarke to this laboratory enabled studies on the suitability of this organism for the assay of itaconate and CAD to be performed. Initial investigations demonstrated that the organism was capable of growth on itaconate as the sole source of carbon, and an extract of cells grown in this way catalysed stoichiometric conversion of itaconate to pyruvate phenylhydrazone in the presence of standard concentrations of ATP, CoA, Mg^{2+}, phenylhydrazine and phosphate buffer. Thus, as would be expected from the known route of itaconate dissimilation, which produces a 2- and a 3-carbon compound, an intact glyoxylate cycle

appeared to be unnecessary for growth on itaconate.

Nevertheless, assay of isocitrate lyase activity in a cell-free extract of itaconate-grown Pseudomonas aeruginosa PAC 501 led to the discovery that the enzyme was present. However, although the specific activities of the itaconate-consuming systems in the wild-type and mutant organism were comparable, the specific activity of isocitrate lyase in the mutant was found to be only 8% of that shown to be present in the wild-type. Thus, although the ratio of the specific activities of cis-aconitate-to-glyoxylate : itaconate-to-pyruvate was 3 : 1 in Pseudomonas aeruginosa PA01, the same ratio was 1 : 4 in Pseudomonas aeruginosa PAC 501. The mutant organism was, therefore, twelve times more suitable for the assay of CAD than was the wild-type.

New Standard Conditions for the Determination of Itaconate

In the experiments described henceforth, the standard conditions for itaconate assay were as previously described (p.55) with the modification that the itaconate-grown Pseudomonas extract employed was prepared from Pseudomonas aeruginosa PAC 501.

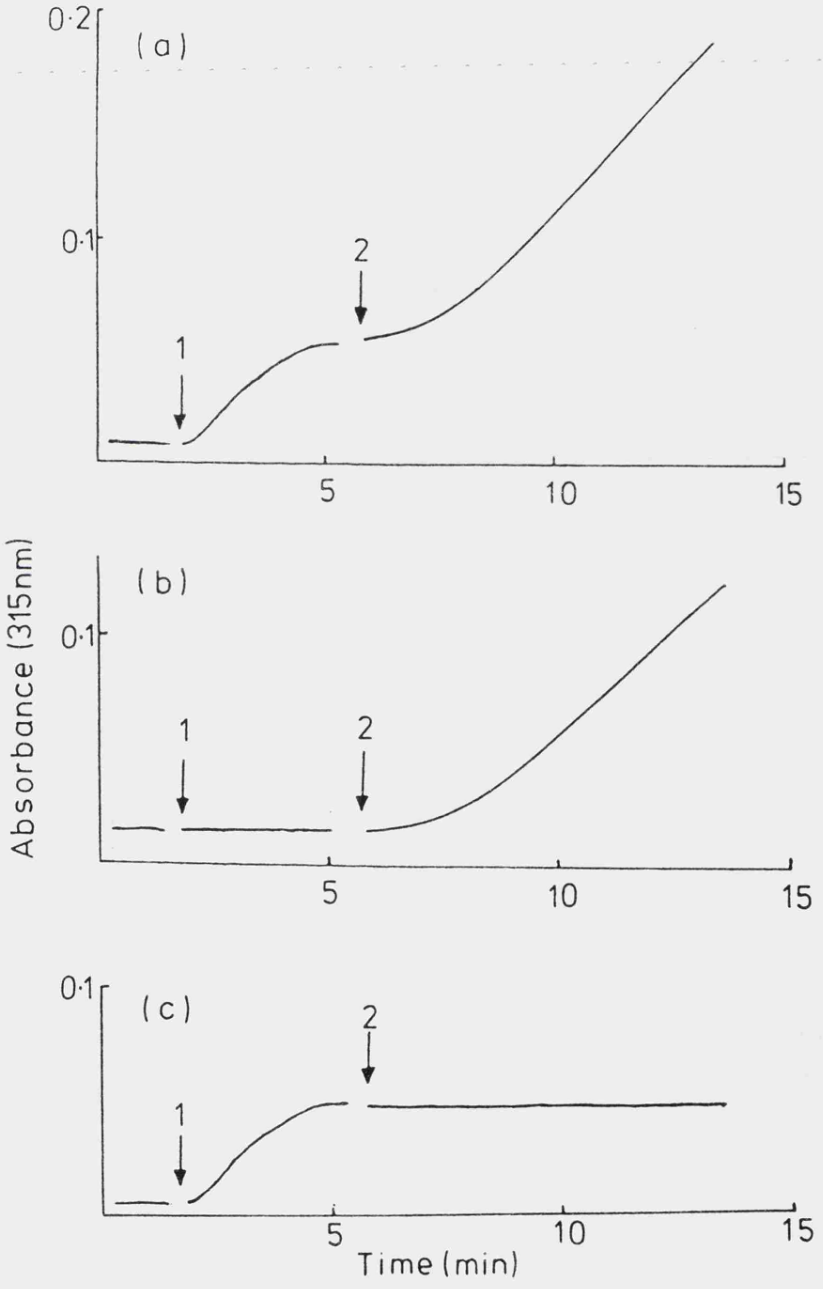
Detection of CAD Activity

An extract of itaconate-producing Aspergillus terreus (50µl) was added to the standard itaconate assay,

containing 20µl of Pseudomonas extract, and the absorbance at 315nm was monitored against a duplicate blank cuvette from which the CoA and ATP had been omitted. Copies of the spectrophotometer output are shown in Fig. 7. Immediately following the addition of the Aspergillus extract, an increase in absorbance was observed which took place over 3-4min, the extent of which was shown to be proportional to the volume of Aspergillus extract added. No such reaction was observed if the Aspergillus extract had been passed through a Sephadex G-25 column prior to addition (Fig. 7b). These results indicate that the response was caused by endogenous itaconate present in the Aspergillus extract.

Subsequent addition of 50µl of 100mM disodium cis-aconitate (to both cuvettes) led to the development (after a 2-3min lag period) of a linear rate of increase in absorbance. This reaction was not observed if the Aspergillus extract had been heated to 90°C for 5min and then cooled, prior to addition (Fig. 7c). Furthermore, the initial rate of the reaction was shown to be directly proportional to the volume of added Aspergillus extract, over the range shown in Fig. 8. Addition of larger volumes of extract resulted in a deviation from linearity caused by saturation of the linking enzymes. This could be alleviated to some extent by the addition of more Pseudomonas extract, but the accuracy of the assay then diminished at the resulting higher absorbances. Neither

FIG. 7. Spectrophotometer traces showing pyruvate phenylhydrazone formation following various additions to the itaconate assay system:
(a) ASPERGILLUS extract (1) followed by CIS-aconitate (2);
(b) desalted ASPERGILLUS extract followed by CIS-aconitate (2);
(c) deproteinized ASPERGILLUS extract followed by CIS-aconitate (2).



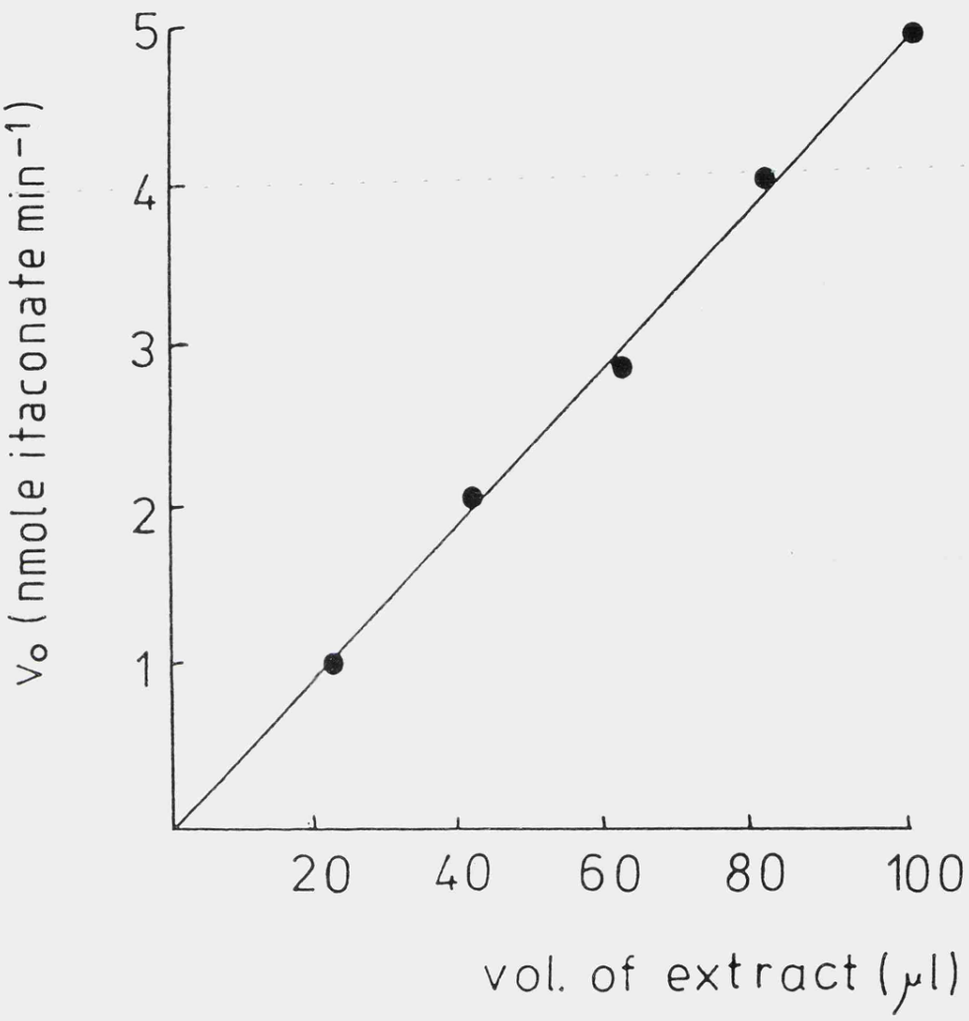


FIG. 8. Relationship between the initial rate of itaconate formation from CIS-aconitate and the volume of ASPERGILLUS extract added to the assay system.

the 'endogenous' reaction nor the response to cis-aconitate were obtained when an extract of non-itaconate-producing Aspergillus was employed. The above results thus indicate the enzymic conversion of cis-aconitate to itaconate in an extract of itaconate-producing Aspergillus.

Substituting identical concentrations of citrate and isocitrate for cis-aconitate in the assay system led to the development of slower initial rates (Fig. 9), thus suggesting that cis-aconitate is a more immediate precursor of itaconate than are citrate or isocitrate. In vitro conversion of citrate and isocitrate to cis-aconitate will have been facilitated by the combined activities of Pseudomonas and Aspergillus aconitases present in the extracts.

These findings coincide with those reported by Bentley and Thiessen (1957a) who performed similar experiments to demonstrate manometrically the enzymic decarboxylation of cis-aconitate in cell-free extracts of Aspergillus terreus (NRRL 1960). These authors proposed the existence of the enzyme CAD, and the results presented above confirm the presence of this enzyme in itaconate-producing mycelia.

Standard Conditions for the Continuous Assay of CAD

All subsequent experiments in which the activity of CAD was determined continuously were performed under the following conditions. The sample cuvette contained 20mM sodium-potassium phosphate buffer, pH 7.0, 10mM phenylhydrazine, 2mM ATP, 0.2mM $MgCl_2$, 0.2mM CoA and 20µl of extract of itaconate-grown Pseudomonas aeruginosa (PAC 501) (approximately 0.4mg of soluble protein). The reference cuvette contained all the above except ATP and CoA. The absorbance at 315nm was followed in a dual-beam spectrophotometer at 25°C. After the above mixtures had been constituted, the sample of Aspergillus extract to be assayed for CAD activity was added, one aliquot to each cuvette. The subsequent endogenous reaction, which caused a small increase in absorbance, was allowed to proceed to completion and 50µl of 100mM disodium cis-aconitate was then added to each cuvette (total volume, 1ml). Following the brief period of hysteresis, the initial rate of the reaction was determined. One unit of CAD activity was defined as that amount of enzyme causing the conversion of 1µmole of cis-aconitate to itaconate per minute, under these conditions.

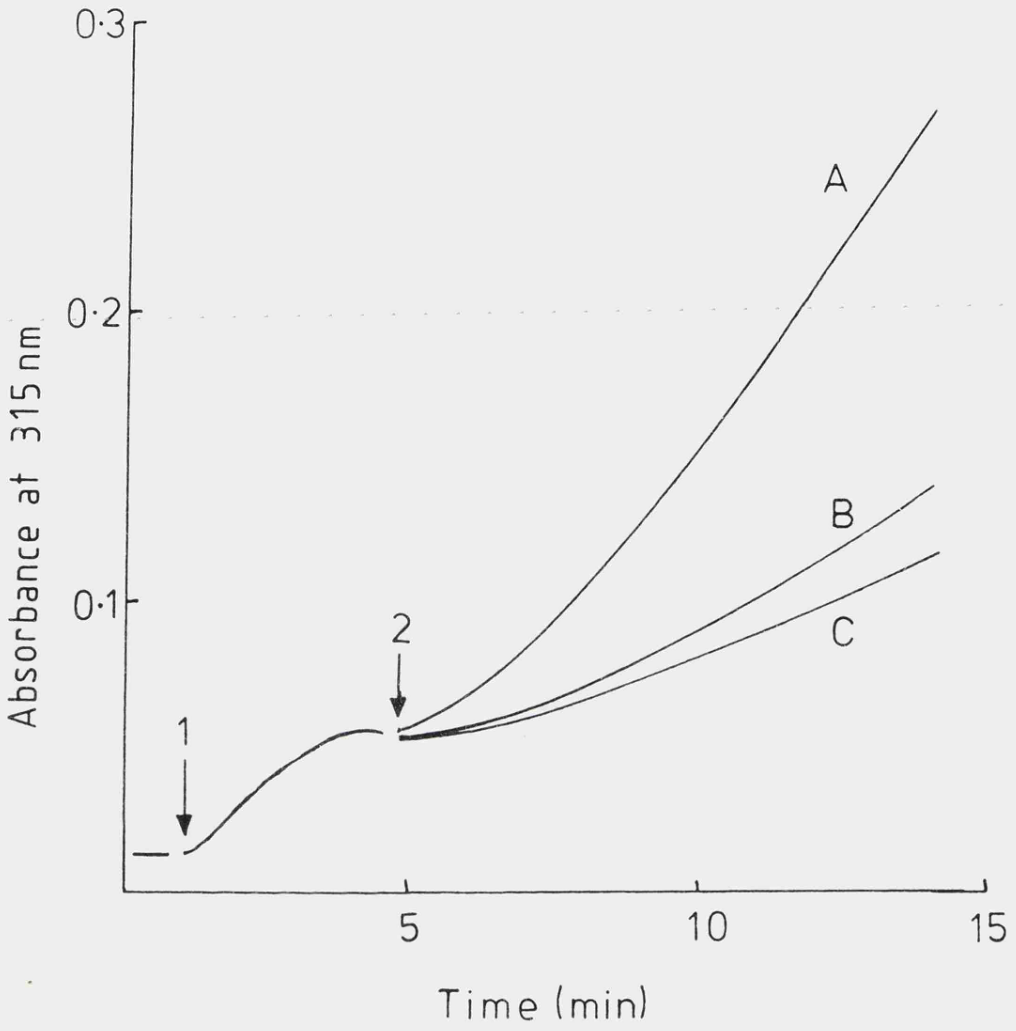


FIG. 9. Comparison of the initial rates of itaconate formation from CIS-aconitic (A), DL-isocitric (B) and citric (C) acids. Sodium salts of these acids (5μmoles in each case) were added to the itaconate assay system at the points indicated by the second arrow. The first addition (1) was 50μl of unfractionated itaconate-producing ASPERGILLUS extract (approximately 0.2mg of protein).

DISCONTINUOUS ENZYME-LINKED ASSAY FOR CAD USING *PSEUDOMONAS AERUGINOSA* (PAC 501)

The presence of aconitase in the *Pseudomonas* linking system precluded the use of the continuous assay for a more detailed analysis of CAD. In addition, the preliminary experiments showed that the linking system became significantly less active as the pH was reduced below 6.5. The CAD assay was therefore performed discontinuously with a partially-purified extract of *Aspergillus*. The procedure for the 40-60% ammonium sulphate fractionation (described in Materials and Methods) resulted in a 3-fold purification of CAD with only 15% loss of total activity; moreover, this salt fractionation resulted in the complete removal of aconitase, which remained in the supernatant at 60% saturation.

A More Sensitive Standard Curve for Itaconate

The standard curve for itaconate shown in Fig. 2 demonstrated the enzyme-linked assay to be effective over the range of 20-100nmoles of itaconate. In Fig. 10, the sensitivity of the assay was extended to measure between 1 and 10 nmoles of itaconate accurately. This more sensitive standard curve was constructed as described for Fig. 2 with the exception that *Pseudomonas aeruginosa* PAC 501 was employed instead of the wild-type PA01; it was used to determine the itaconate produced enzymically in

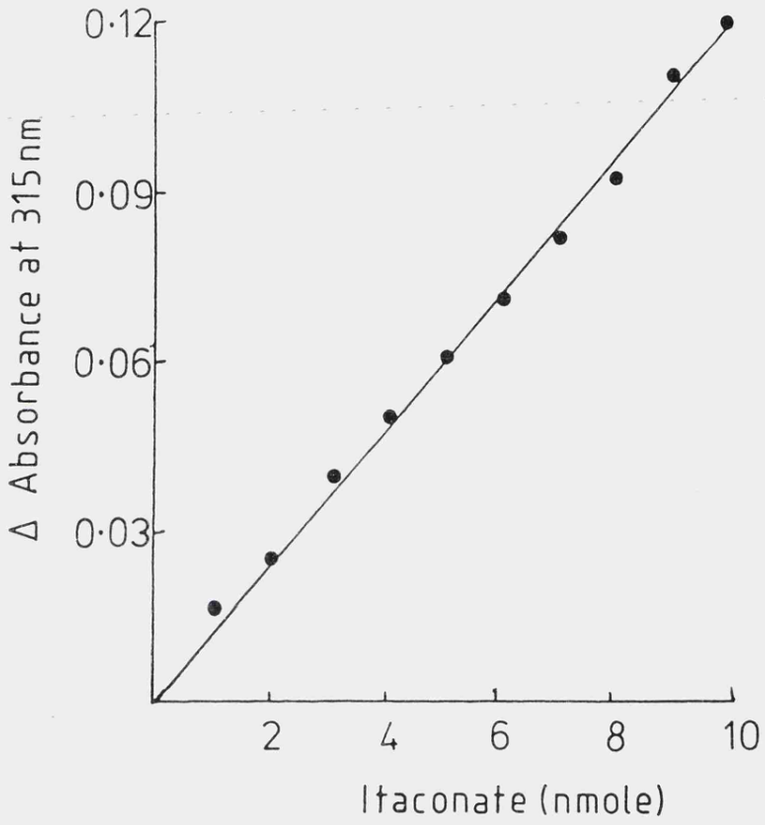


FIG. 10. Standard curve for the detection of between 1 and 10nmoles of itaconate.

incubation mixtures of Aspergillus extract and cis-aconitate.

Standard Conditions for the Discontinuous Assay of CAD

Mixtures containing Aspergillus extract and disodium cis-aconitate (amounts as indicated) in 20mM sodium-potassium phosphate buffer, pH 6.0, to a total volume of 3ml were incubated in a water-bath at 30°C; reactions were started by the addition of the extract. At selected times, 0.25ml aliquots were withdrawn and immediately transferred to pre-heated test-tubes in a boiling water-bath for 3min, after which the precipitated protein was removed by centrifugation in a microfuge. The supernatant solutions were then assayed for itaconate under the standard conditions described for the continuous assay of CAD (see p.78) in which 20-100µl of sample were added to each cuvette, in place of Aspergillus extract and substrate, and the extent of the subsequent increase in absorbance was determined.

Time-Course Experiments

Figure 11 shows a time-course for the conversion of cis-aconitate (initial concentration, 1mM) to itaconate, catalysed by CAD present in the 40-60% ammonium sulphate fraction. After two hours, the concentration of itaconate in the incubation mixture was 0.97mM, indicating almost complete conversion. Therefore, under these conditions, the CAD reaction appeared to be

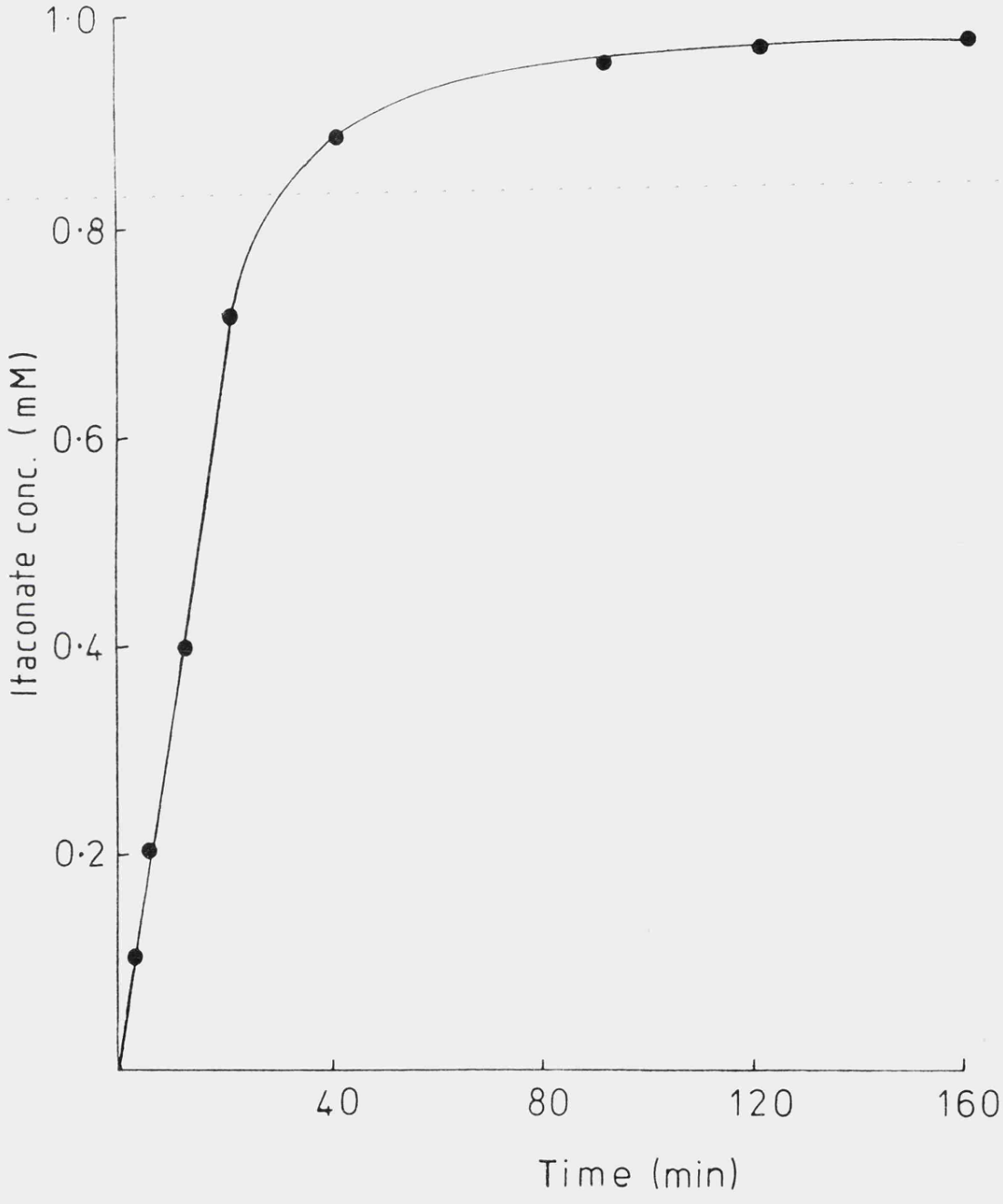


FIG. 11. Time-course for the conversion of CIS-aconitate (initial concentration 1mM) to itaconate, catalysed by CAD.

essentially irreversible.

A similar time-course experiment was performed in which a higher concentration of cis-aconitate was used in conjunction with a smaller amount of CAD. The details are given in Fig. 12 which shows that, for at least the initial 30min of the reaction, the rate of itaconate formation was constant. The elucidation of these conditions was important for the subsequent experiments which required the determination of initial reaction velocities.

The Determination of the pH Optimum of CAD

CAD activity was assayed in a range of buffer solutions. The incubation mixtures contained 10mM cis-aconitate and 20µl of the Aspergillus ammonium sulphate fraction. Sodium-potassium phosphate buffers (20mM), pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, and sodium acetate - acetic acid buffers (20mM), pH 4.0, 4.5 and 5.0 were employed. Aliquots were withdrawn at 2min intervals for the first 10min of the reaction, and were then assayed for itaconate. The results are shown in Fig. 13 and are presented in Fig. 14 as a plot of CAD activity against pH. The pH optimum of CAD was found to be approximately 5.5. CAD was 40% less active in the acetate buffer, and at pH 4.5 there was no detectable activity. Also significant is that the enzyme was 50% less active at pH 7.0 (the pH at which the continuous assays were

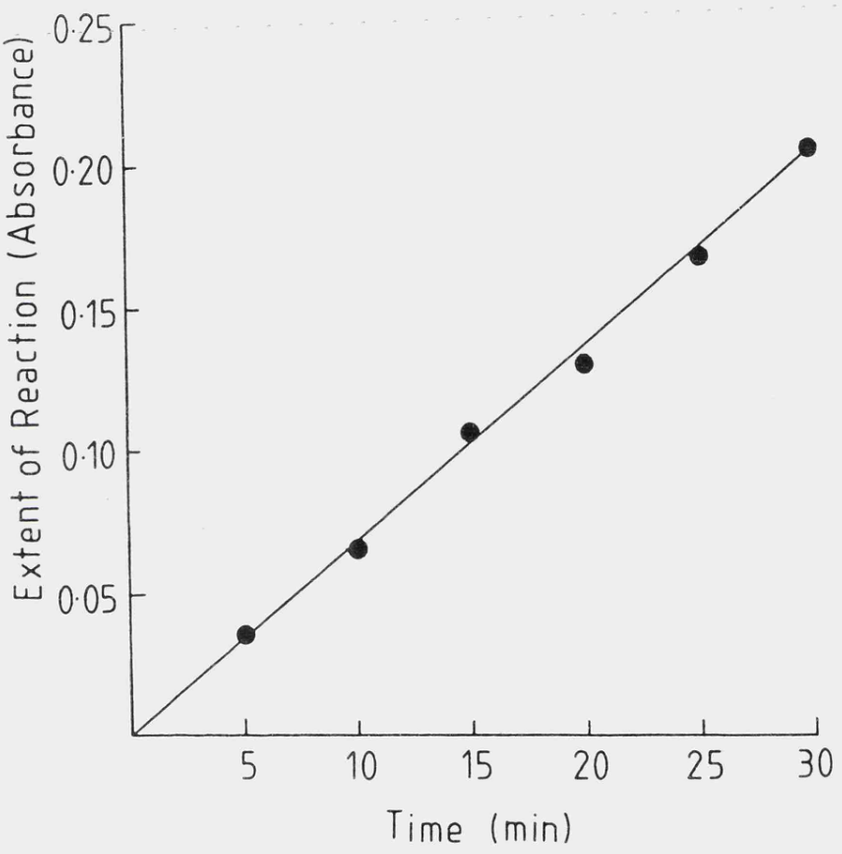


FIG. 12. Time-course for the conversion of CIS-aconitate (initial concentration 20mM) to itaconate, catalysed by CAD. To the standard discontinuous CAD assay system were added 20µl of a 40-60% ammonium sulphate fraction of ASPERGILLUS extract and 600µl of 100mM disodium CIS-aconitate.

FIG. 13. CAD activity time-courses in a range of buffer solutions.

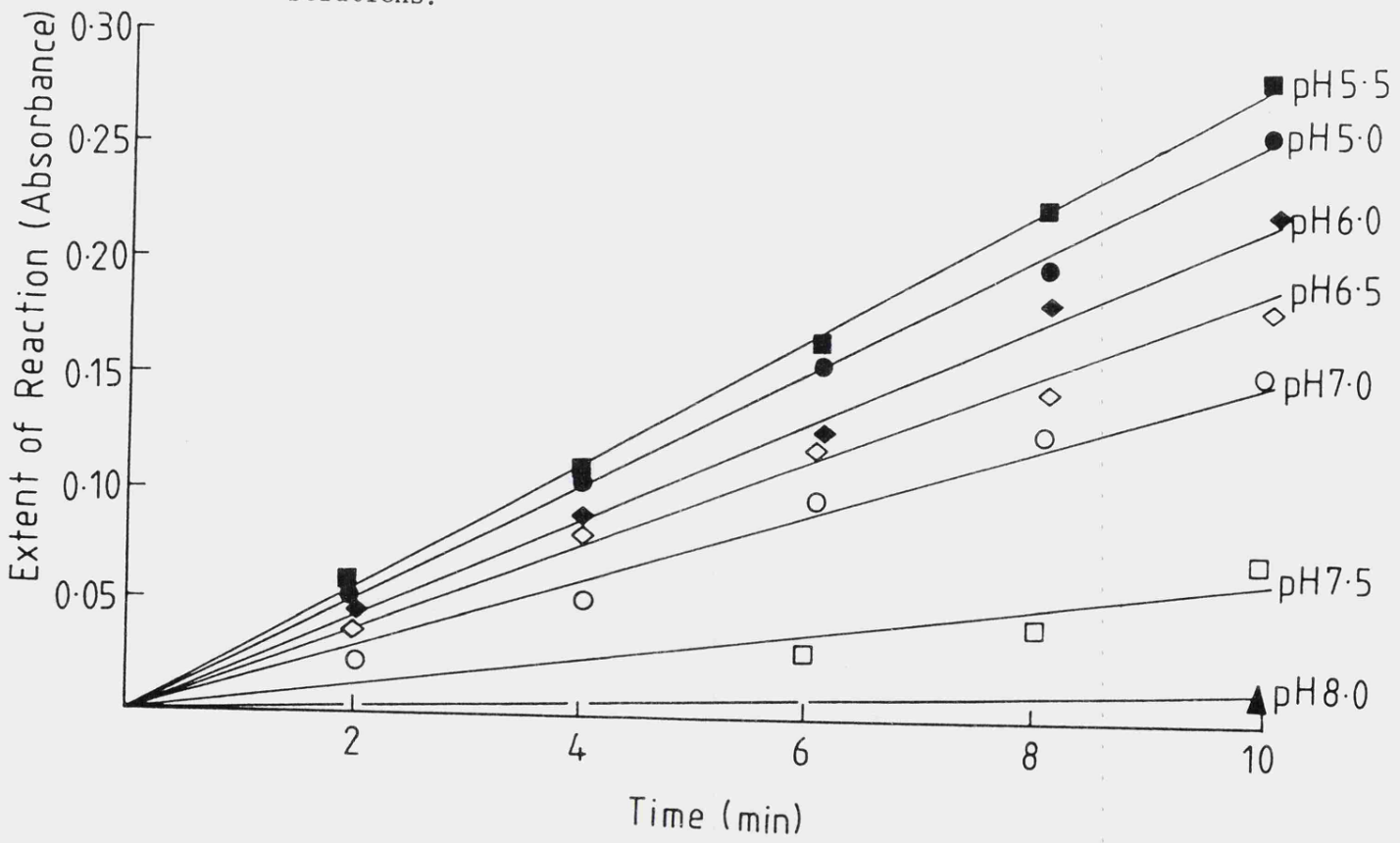
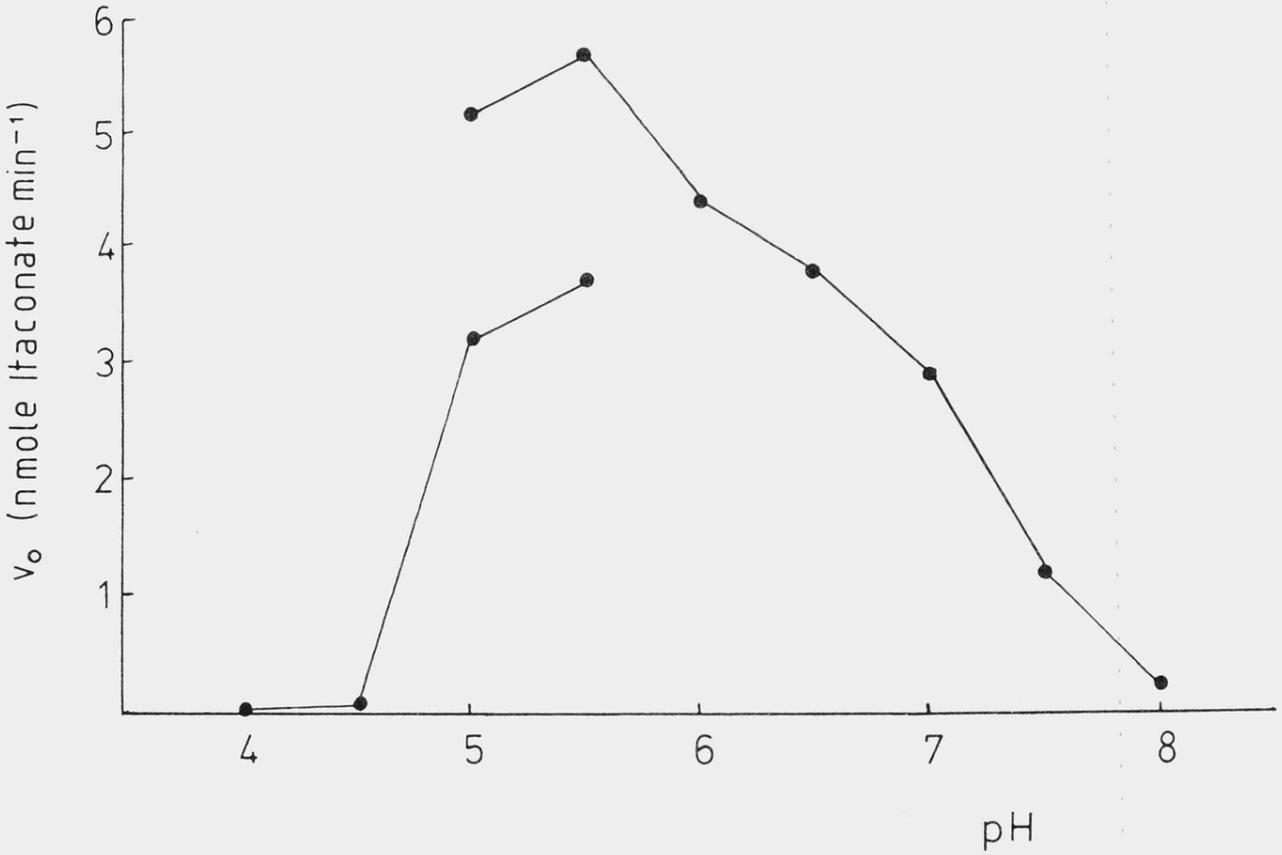


FIG. 14. pH profile for CAD activity.

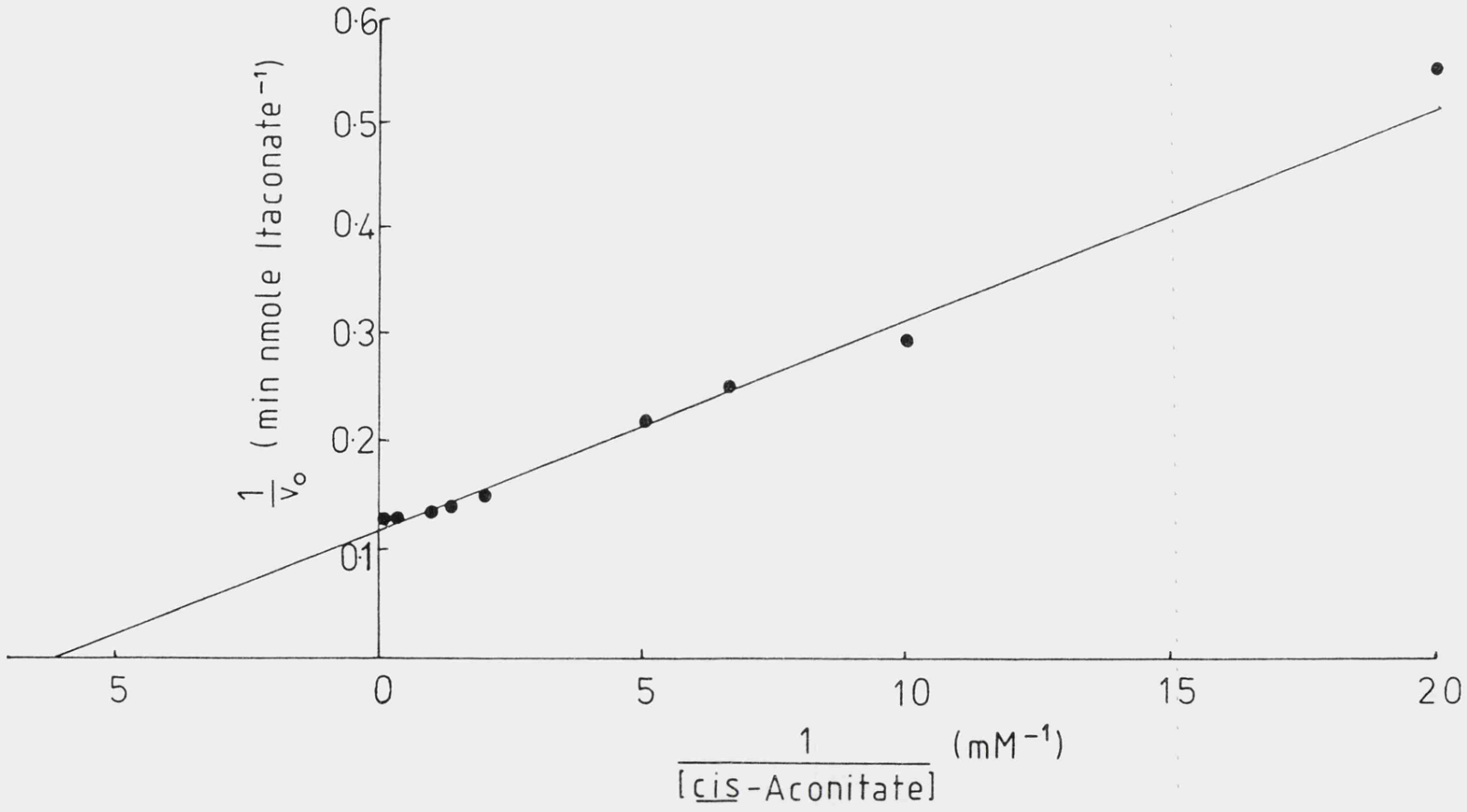


performed) than at pH 5.5.

The Determination of the Km of CAD for cis-Aconitate

Initial rates were determined, as described above, over a range of concentrations of cis-aconitate (from 0.05mM to 15mM) at pH 6.0. Figure 15 presents the data in the form of a double-reciprocal plot from which the Km value was determined to be 0.15mM. This value was confirmed by analysis of the data using the computer-fitted direct linear plot method of Eisenthal and Cornish-Bowden (1974).

FIG. 15. Double-reciprocal plot for the determination of the Km of CAD for CIS-aconitate.



ENZYME AND NUTRIENT CHANGES DURING THE FERMENTATION OF ASPERGILLUS TERREUS

The presence of CAD in itaconate-producing Aspergillus terreus and its apparent absence from non-itaconate-producing mycelia was a strong indication of the involvement of the enzyme in itaconate synthesis. A closer investigation of the importance of CAD was facilitated by following its activity throughout a fermentation of Aspergillus terreus. Such studies were considered especially significant in view of the reported decrease in NADP-dependent isocitrate dehydrogenase activity during acid production (Winskill, 1983). Analyses of the specific activities of CAD, citrate synthase, aconitase and NADP-dependent isocitrate dehydrogenase in relation to each other and to the synthesis of itaconate were therefore performed.

Replicate-flask cultures of itaconate-producing Aspergillus terreus, inoculated with spores washed from Czapek-Dox agar slopes, were harvested at selected times and analysed for mycelial growth, itaconate production and enzyme activities. The results are presented in Fig. 16. The long lag period of 60 hours, prior to the period of accelerated growth, is indicative of a low inoculum concentration. However, a separation of the accelerated-growth phase from the acid-producing phase

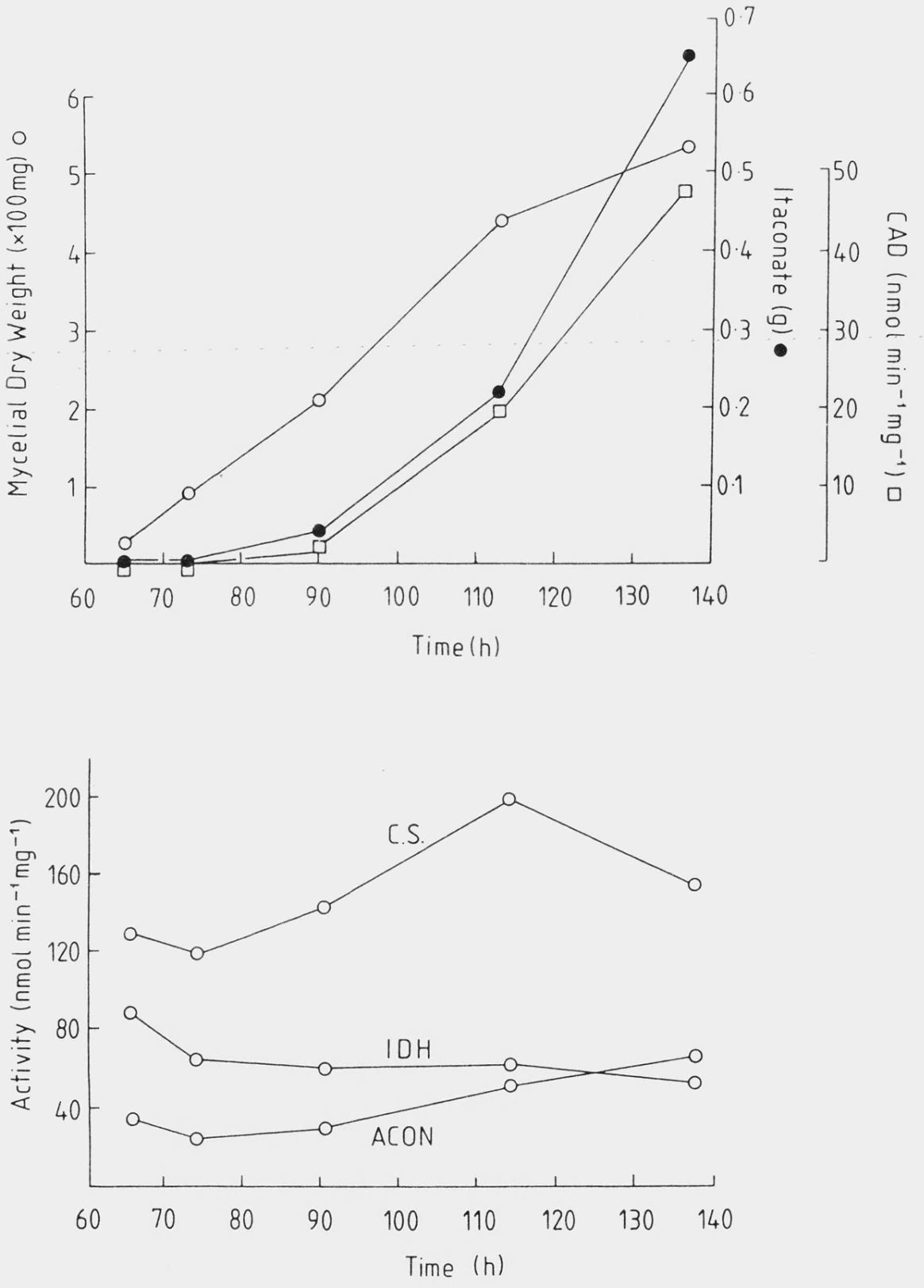
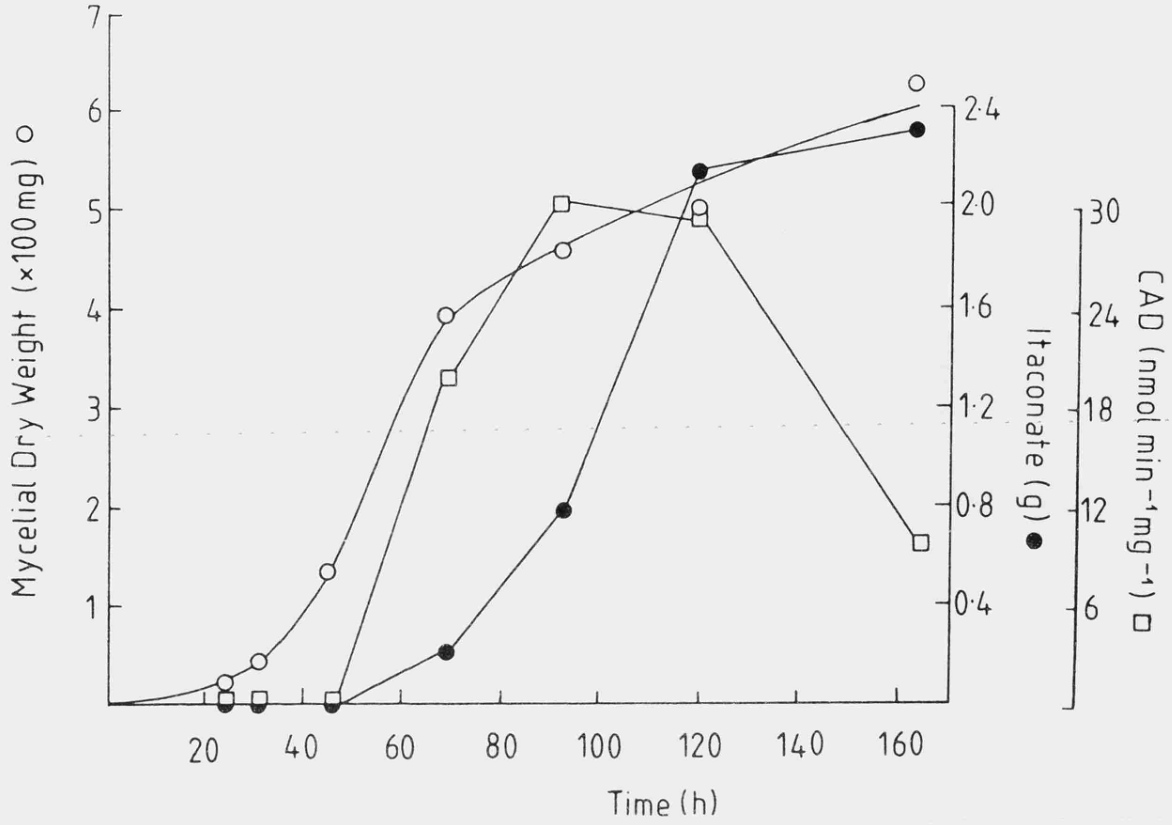


FIG. 16. Time-course for the fermentation of ASPERGILLUS TERREUS in replicate flask cultures showing itaconate production and accompanying changes in enzyme activities.

was clearly obtained; acid production started approximately 90 hours into the fermentation. During the initial period of growth, there were no significant changes in the detected levels of citrate synthase, aconitase or NADP-dependent isocitrate dehydrogenase. Furthermore there appeared to be no intracellular CAD activity associated with this growth phase. Between 90 and 140 hours, however, during which time the rate of biomass production decreased, there was a 100-fold increase in the specific activity of CAD. This was accompanied by a notable increase in the extracellular concentration of itaconate. Thus, there appeared to be an intimate association between the intracellular activity of CAD and the production of itaconate. In addition, during this acid-production phase, the specific activities of the three citric acid cycle enzymes citrate synthase, aconitase and NADP-dependent isocitrate dehydrogenase appeared to remain unchanged.

In subsequent experiments, the fermentation was followed to the completion of the acid-production phase. In order to decrease the length of time prior to exponential growth, a heavier inoculum was employed. Replicate flasks were inoculated (2% v/v) with a 5-day flask culture as described in the Materials and Methods section. The results of the analysis are shown in Fig. 17. The lag period was reduced to less than 20 hours; the subsequent period of exponential growth was



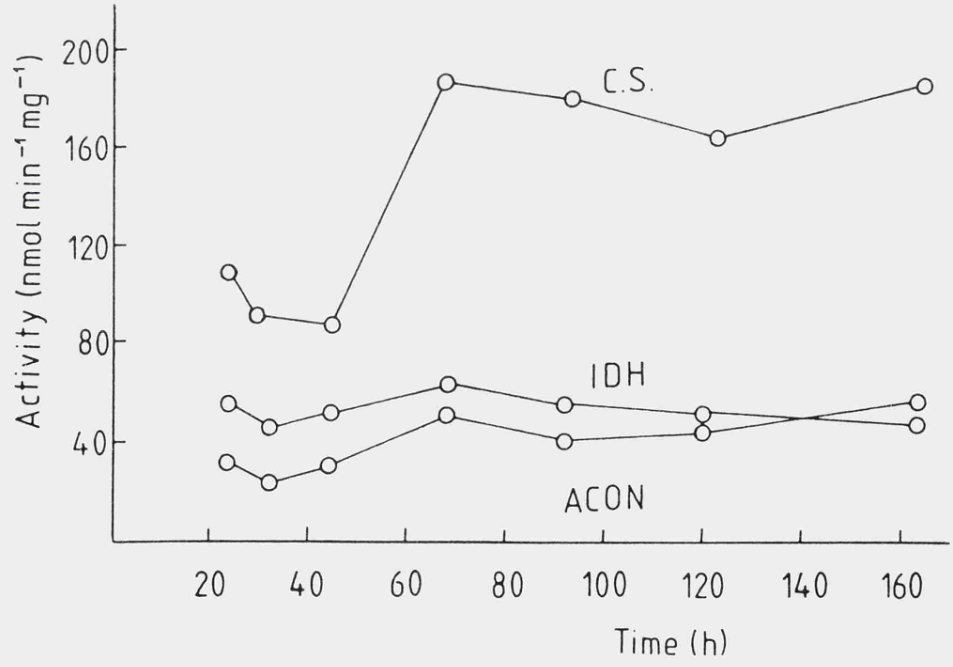


FIG. 17. Profile of itaconate production by ASPERGILLUS TERREUS. A heavier inoculum was used than in Fig. 16 and the fermentation was followed to completion.

over at 6o hours and was followed by a small linear rate of increase in growth. Between 45 and 90 hours, there was a steady rise in intracellular CAD activity which then remained constant until 120 hours. This period coincided precisely with the acid-production phase, and, between 120 and 170 hours, the decrease in the rate of itaconate production was accompanied by a reduction in intracellular CAD activity. There were no significant changes in citrate synthase, aconitase or NADP-dependent isocitrate dehydrogenase throughout. These results provide further evidence for the close association between CAD activity and itaconate production.

The periods of growth and acid synthesis therefore appeared to follow the classical pattern associated with secondary metabolite production. During unlimited growth, when all the nutrients necessary for biomass production were present in excess, no itaconate production was observed. However, when the period of exponential growth was over, and the rate of biomass production was decreasing, itaconate began to appear in the medium. During this acid-producing period, biomass formation did not cease completely but continued at an approximately linear rate. Such an observation may be indicative of growth being limited by the availability of one or more nutrients.

Fermenter Studies

Although the above studies proved to be effective in the investigation of key features of the itaconate fermentation, they were limited by the nature of the culture conditions. Small errors in the distribution of the inoculum, for example, led to larger discrepancies between the individual flask cultures. The fermentation was therefore followed subsequently in a 3-litre laboratory fermenter. Under such conditions, the uniformity of the culture was maintained more easily. The results of a fermentation, in which a 2% (v/v) inoculum of a 5-day flask culture of Aspergillus terreus was employed, are shown in Fig. 18. The salient features to be noted are identical to those described above for the replicate-flask fermentation. However, the greater flexibility of the fermenter system was put to further use in subsequent experiments; in addition to following itaconate production, growth and individual enzyme activities, the levels of carbon, nitrogen and phosphorus present in the culture medium were also monitored.

Standard curves for the determination of sucrose, ammonia and phosphorus are presented in Figs. 19, 20 and 21 respectively. When the levels of these nutrients present in the culture medium were superimposed on a fermentation profile, as shown in Fig. 22, it became clear that sucrose and ammonia were present in excess in the culture medium throughout. However, only 35 hours after

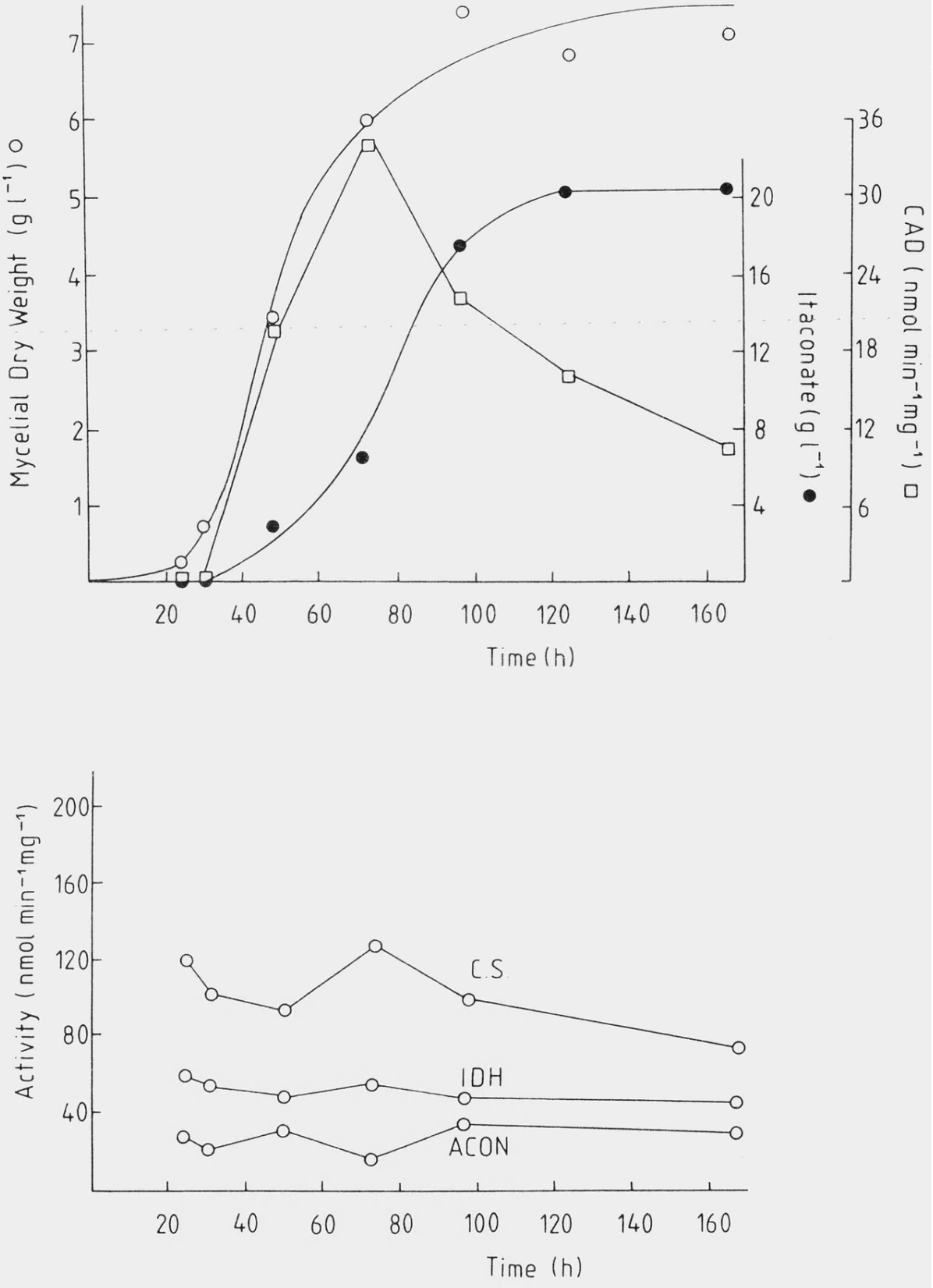


FIG. 18. Profile of itaconate production by ASPERGILLUS TERREUS in the 3-litre fermenter.

FIG. 19. Standard curve for the determination of sucrose.

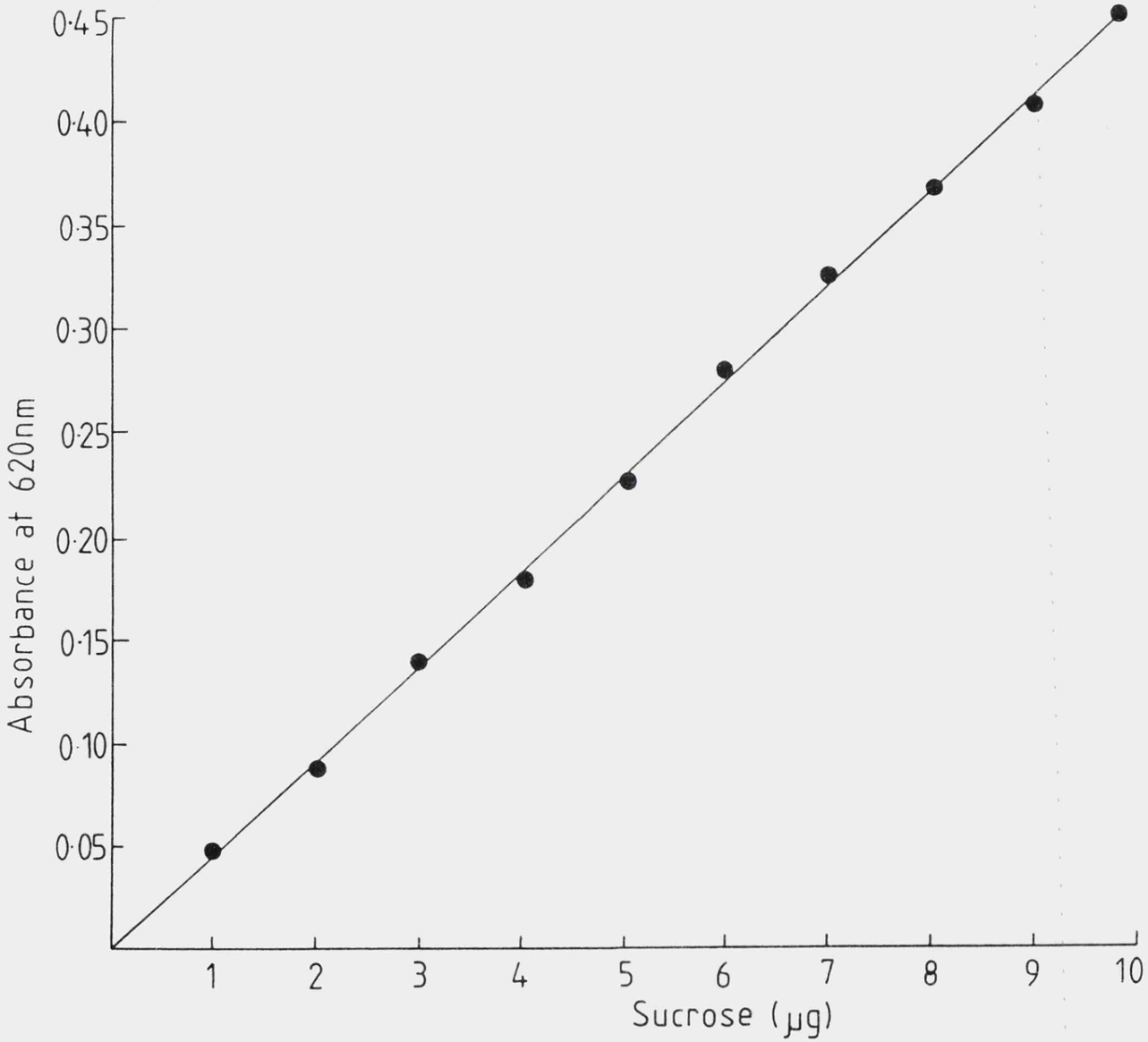


FIG. 20. Standard curve for the determination of ammonia.

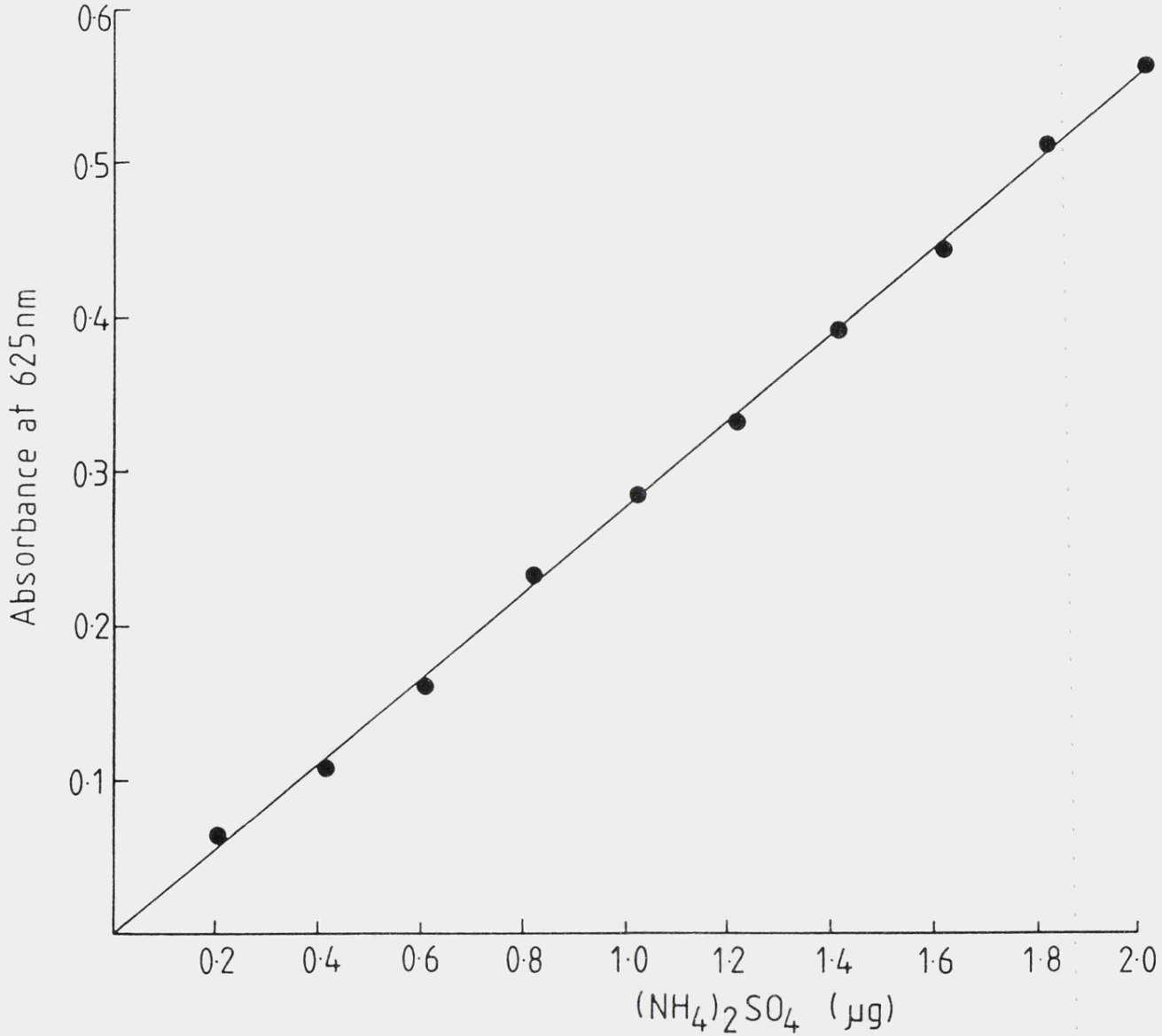
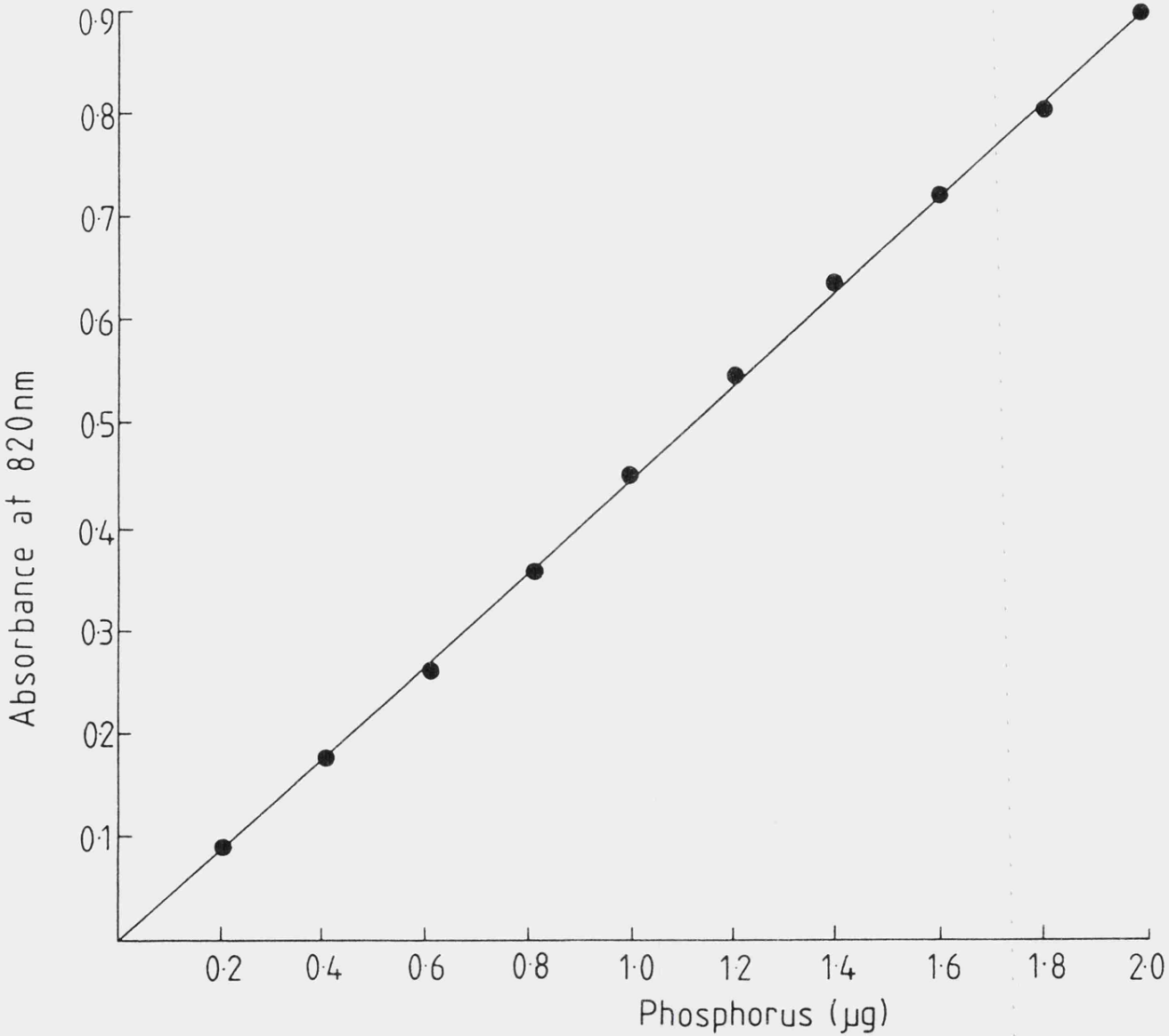


FIG. 21. Standard curve for the determination of phosphorus.



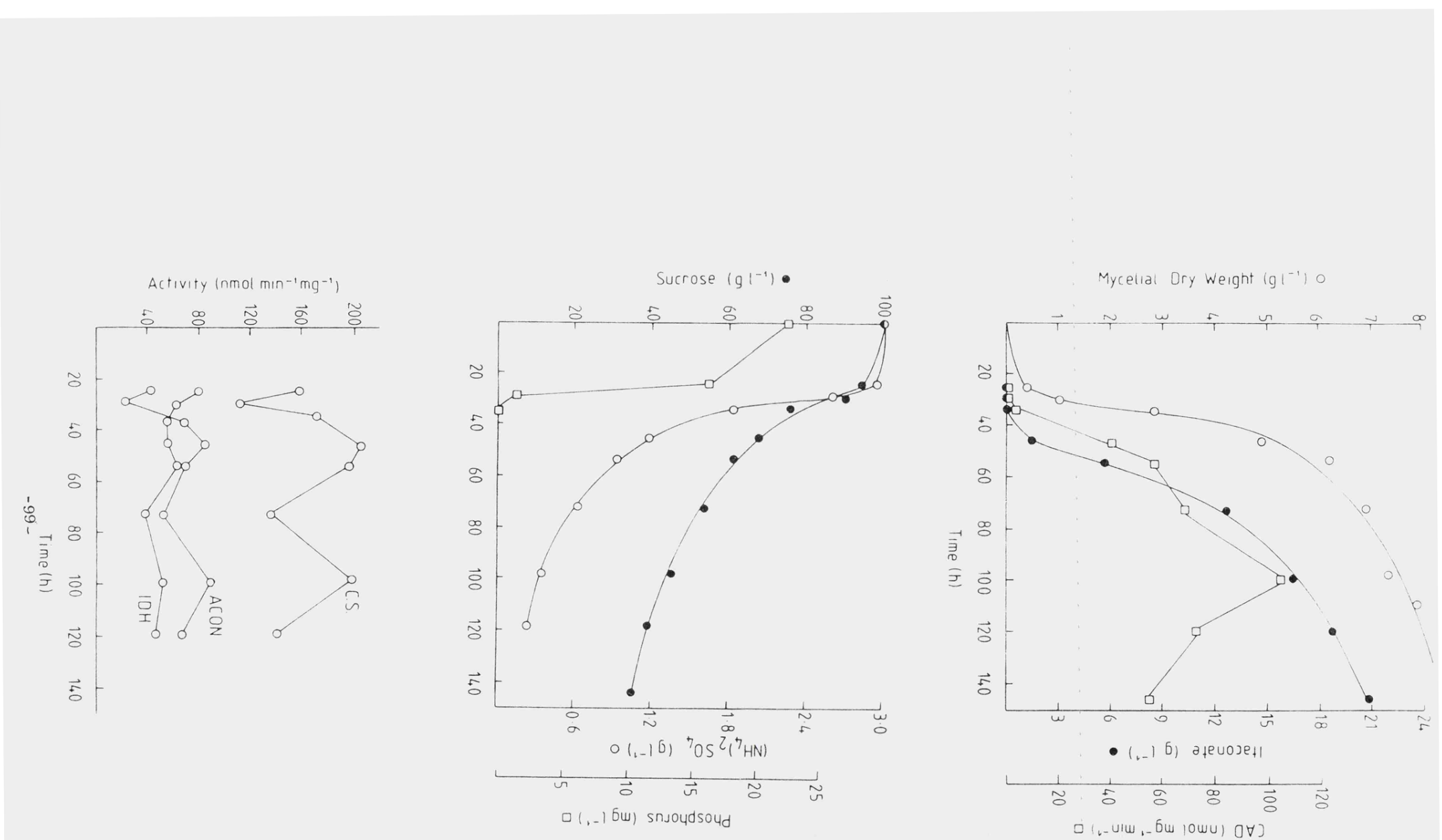


FIG. 22. Profile of itaconate production by ASPERGILLUS TERREUS in the fermenter, also showing changes in nutrient levels and enzyme activities.

inoculation, there was no detectable phosphate in the culture medium. This finding is especially significant when it is seen that within the subsequent 10 hours the intracellular level of CAD activity increased from almost zero to approximately 35% of its maximal value. This period also coincided precisely with the first appearance of itaconate in the culture medium. It therefore appears that the end of the period of exponential growth and the onset of itaconate production (facilitated by the appearance of intracellular CAD) could be triggered by exhaustion of phosphate from the culture medium. This observation was repeated when the same experiment was performed in replicate shake-flask cultures, as shown in Fig. 23. As with the previous fermentations, there were no significant changes in the activities of citrate synthase, aconitase or NADP-dependent isocitrate dehydrogenase.

Quantitation of the Rate of Itaconate Production

The results presented thus far in this section confirm the close association between itaconate production and CAD activity and serve to indicate the importance of the concomitant depletion of phosphate from the culture medium. The purpose of the following experiment was to compare the specific rate of itaconate production with the specific activity of CAD detected in the Aspergillus mycelium. This was in order to discover whether the detected level of CAD activity was sufficiently high to

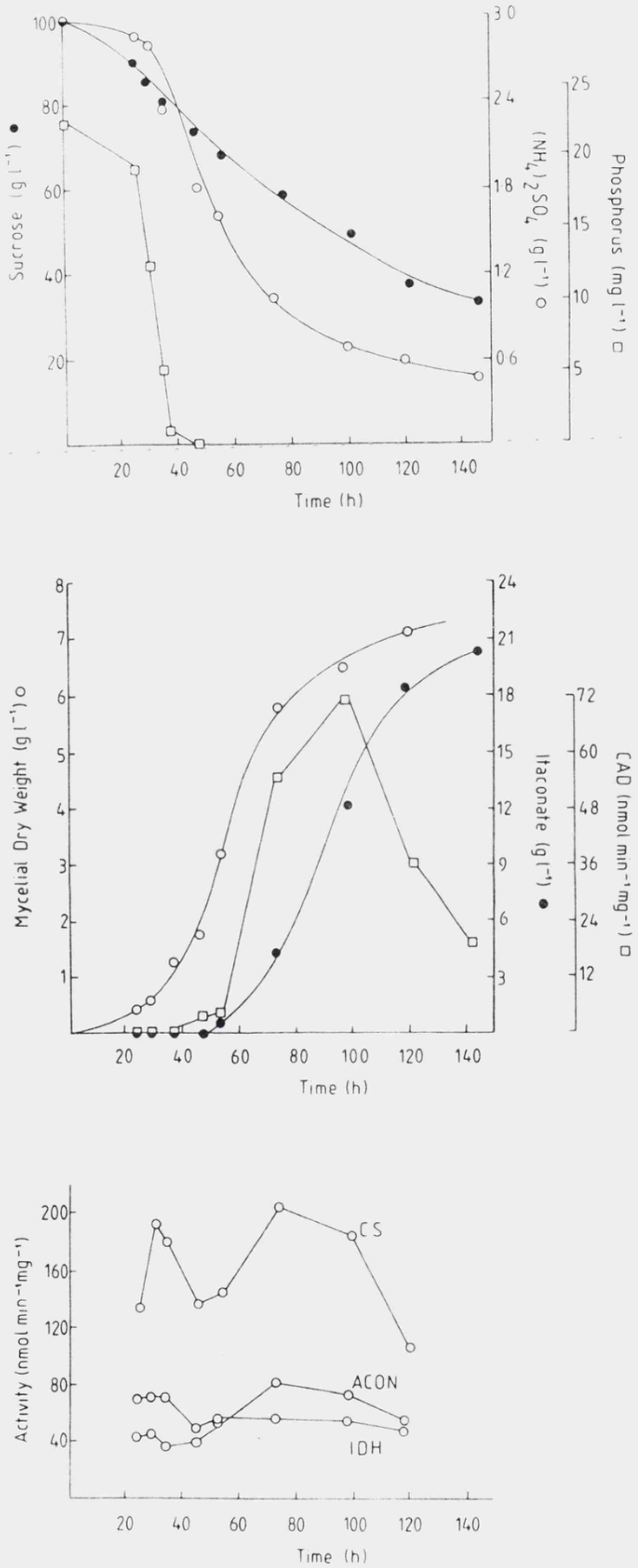


FIG. 23. Profile of itaconate production by ASPERGILLUS TERREUS in replicate-flask cultures, showing the same analyses as in Fig. 22.

account for the amount of itaconate produced. Throughout this report, the specific activity of CAD has been expressed in units of nmoles min^{-1} mg^{-1}protein. Consequently, in order to compare the specific activity of CAD with the specific rate of acid production, the latter has to be expressed in these units. In the fermentation, the rate of itaconate formation (dp/dt) was related to the amount of biomass (x) in the following way:

$$\frac{dp}{dt} = q_p.x$$

where q_p = the specific rate of itaconate formation. It therefore follows that:

$$\int_{p_1}^{p_2} dp = q_p \int_{t_1}^{t_2} x.dt$$

and:

$$q_p = \frac{p_2 - p_1}{\int_{t_1}^{t_2} x.dt} \qquad \text{eqn. 1.}$$

The numerator of equation 1 was calculated by fitting the itaconate-formation profile of Fig. 24 to a polynomial equation, with the aid of a computer, and $p_2 - p_1$ values were determined in units of nmole l^{-1} for each hour of the fermentation. Values of the denominator were obtained by measuring the area under the growth curve for each hour of the fermentation and were expressed in units of min.mg protein.l^{-1} after making the assumption that

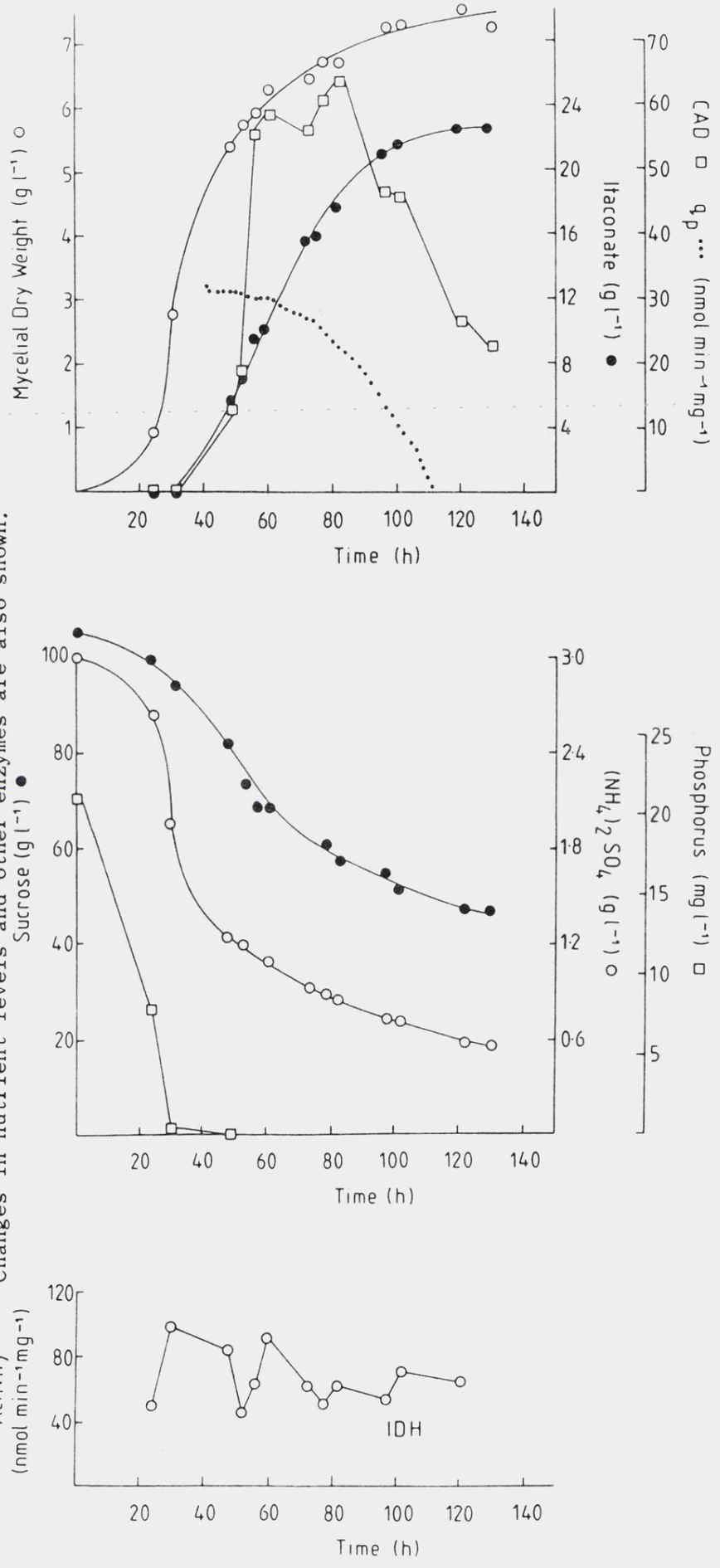


FIG. 24. Comparison of the specific rate of itaconate production (q_p) with the specific activity of CAD in the itaconate fermentation Changes in nutrient levels and other enzymes are also shown.

25% of mycelial biomass existed as soluble protein (Pirt, 1975). The specific rate of itaconate production (q_p) therefore possessed units of nmole min^{-1} mg^{-1} protein and was determined hourly throughout the fermentation. The values of q_p thus obtained are shown in Fig. 24 and may be compared directly with the specific activity of CAD which was measured by direct assay in the continuous enzyme-linked system.

As may be seen in Fig. 24, the values of the specific activity of CAD and q_p lie in the same order of magnitude. For the most part of the fermentation (after 54 hours), there was more than sufficient CAD activity to account for the observed amount of itaconate produced. However, between the start of itaconate production (30 hours) and 54 hours, the q_p value was larger than the specific activity of CAD. Although this discovery may tend to cast doubt on the proposed exclusive role of CAD in the formation of itaconate, it must be realised that CAD activity was assayed at pH 7, and the enzyme had been shown to be twice as active at pH 6. Furthermore, in subsequent studies on the enzyme (to be described), it was discovered that CAD was also twice as active in 20mM triethanolamine buffer, pH 7, as in the 20mM phosphate buffer employed in these assays. These two discoveries indicate that the enzyme may be, *in vivo*, at least four-times more active than was determined *in vitro*. In this case, the specific activity of CAD would be more

than sufficient to account for the specific rate of itaconate formation throughout the duration of the fermentation.

In addition, it may be prudent at this point to list the major assumptions which were made in order to make a direct comparison of q_p and specific enzyme activity:
(i) the V_{max} of the enzyme *in vivo* is greater than, or equal to, the V_{max} determined *in vitro*;
(ii) all of the enzyme was extracted and remained in an active form prior to assay;
(iii) following disruption of the mycelium, all enzyme activity was associated with the soluble protein fraction.

If such conditions were met, an apparent excess of CAD activity over the itaconate which was formed (as was observed in this case after 54 hours) may be easily explained in terms of *in vivo* regulation of activity. For example, it is conceivable that CAD operates in the presence of sub-saturating concentrations of *cis*-aconitate, or that the enzyme activity is subject to some form of allosteric regulation. Clearly, few clear-cut conclusions may be drawn from such an investigation; however, probably the most important observation is that CAD activity *in vitro* and the rate of itaconate production *in vivo* are quantitatively comparable.

Phosphate Pulse

Subsequent to the discovery that the depletion of phosphate from the medium might trigger itaconate production via an intracellular rise in CAD activity, the following experiment was performed in order to discover whether a pulse of phosphate at the time of depletion could prevent (or postpone) itaconate production. The fermenter was inoculated in the usual way and the concentration of phosphate in the culture medium was closely monitored. Mycelial growth, CAD activity, itaconate concentration and ammonia concentration were also followed. After 33 hours, the concentration of phosphate had fallen to approximately 1% of its initial value. At this point in previous fermentations, a large increase in intracellular CAD activity was accompanied by the start of a steady rise in extracellular itaconate concentration. In this experiment, however, a solution containing 0.3g of potassium dihydrogen orthophosphate was added to the fermenter so that the concentration of phosphate in the culture medium was returned to its initial value of 0.1g l^{-1}. The phosphate was allowed to mix with the culture for approximately 1min before a sample was withdrawn. Interestingly, the concentration of phosphate in this sample was found to be 0.05g l^{-1}. Thus, within 1min, the mycelium had taken up 0.15g of phosphate. In spite of the addition of this phosphate, when the next sample was taken the intracellular CAD activity was found to be as high

as in previous fermentations, and the production of itaconate had begun. Moreover, there was no detectable phosphate remaining in the medium.

It therefore appeared that the intracellular mechanisms for secondary metabolite formation had already been activated at 33 hours, and that the subsequent addition of phosphate had no effect on CAD activity and itaconate production. However, between 55 and 69 hours, a deviation from the normal course of events did occur. It appears that the extra mycelial growth, afforded by the supplementary phosphate, caused exhaustion of the ammonia nitrogen source in the medium. All of these changes are shown in Fig. 25. The depletion of ammonia from the medium then caused a rapid fall in intracellular CAD activity; more than 90% of the enzyme activity was lost within the following 14 hours. This was accompanied by a decrease in the rate of itaconate production which fell to zero within 20 hours. The possible significance of these observations is discussed in detail in the General Discussion section.

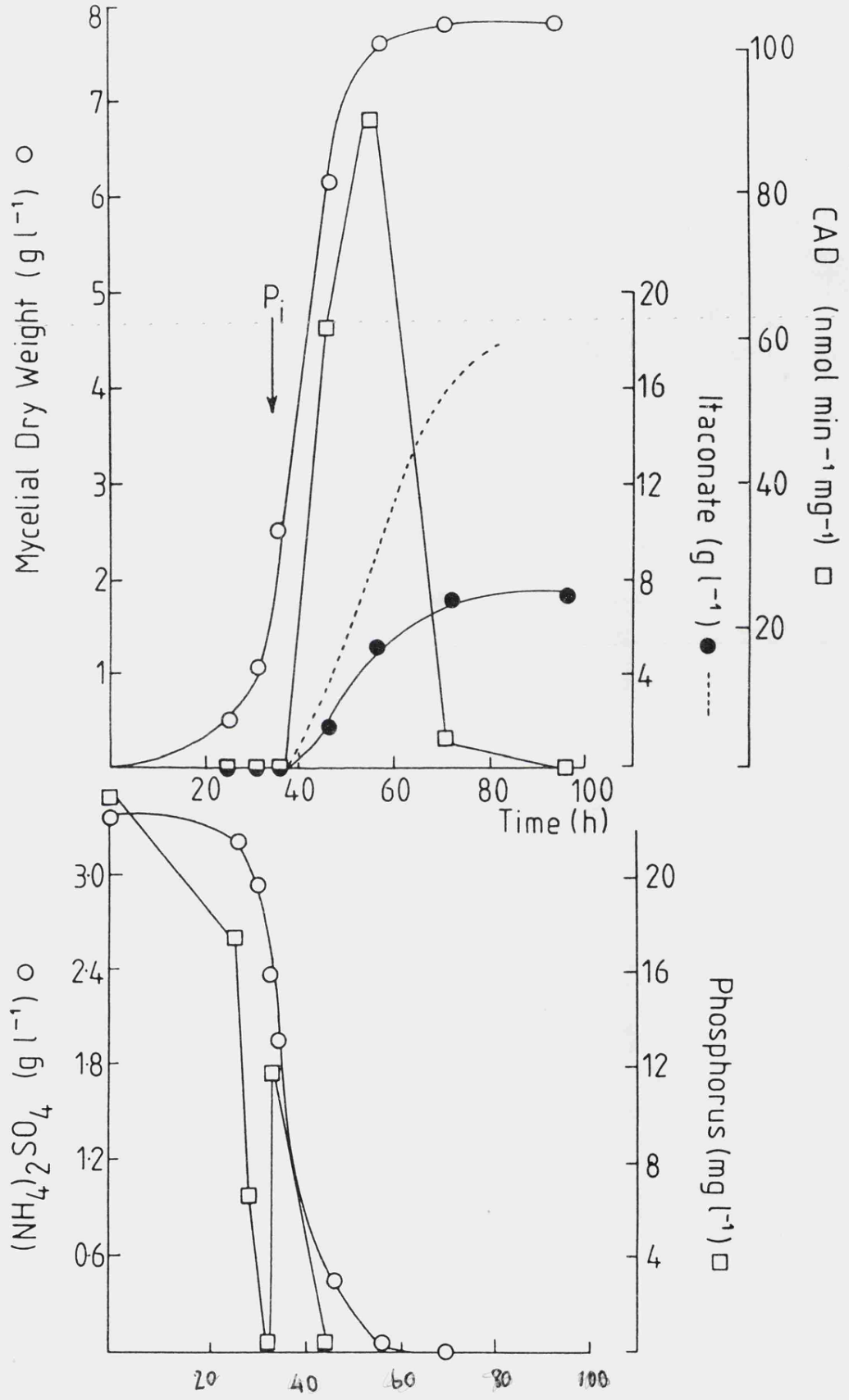


FIG. 25. The effect of phosphate addition, at the point of phosphate exhaustion, to the itaconate fermentation.

SUBSIDIARY EXPERIMENTS

In addition to the experiments described in the preceding pages of the Results and Discussion section, a few subsidiary projects, which are relevant to the general scheme, were begun. These are outlined in the sections below.

Thermal Stability of CAD

Samples (100µl) of the 40-60% ammonium sulphate fraction of CAD were added to pre-incubated glass test-tubes in water-baths operating in a range of temperatures (25, 30, 35, 40, 45, 50 and 55°C). Following 5min incubation, the samples were transferred to an ice-bath and cooled prior to assay. The CAD activity in these samples was then assayed and expressed as a percentage of that activity found in an untreated sample. The results are presented in Fig. 26 and show that the greatest change in activity occurred between the temperatures of 45 and 50°C.

Further Purification of CAD

The 40-60% ammonium sulphate fraction of CAD was subjected to ion-exchange chromatography. Preliminary experiments showed that the enzyme would not bind to carboxymethyl cellulose in 20mM sodium-potassium phosphate buffer, pH 5.5. However, an anionic exchange resin such as DEAE-Sephacel in 20mM triethanolamine buffer, pH7.0, did bind the enzyme. Furthermore, CAD was

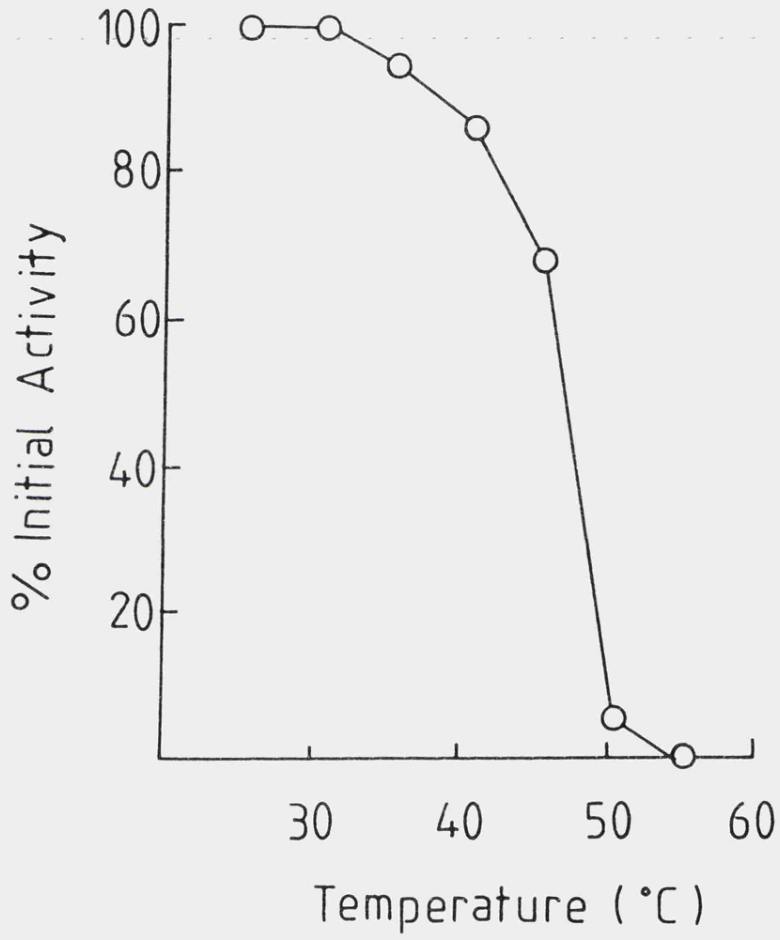


FIG. 26. Thermal stability profile of CAD. Details are given in the text.

shown to be 100% more active in 20mM triethanolamine buffer, pH 7.0, than in 20mM sodium-potassium phosphate buffer, pH 7.0. A solution of the 40-60% ammonium sulphate fraction of CAD was subjected to ion-exchange chromatography using the Fast Protein Liquid Chromatography system as explained in the Materials and Methods section. CAD was eluted at a chloride ion concentration of approximately 15mM. However, the yield of the enzyme from the column (10%) and the purification obtained (less than 2-fold) were disappointing. It is likely that CAD was inactivated by ion-exchange chromatography under these conditions.

Isolation of a Bacterium from Soil

The vigorous cell-disruption techniques which are required to make cell-free extracts of fungal mycelia often preclude further cell and organelle fractionation studies. Thus, the preparation of intact mitochondria from filamentous fungi is difficult, and workers frequently have to be satisfied with very low respiratory control ratios (Nowakowska-Waszczuk, 1973; Winskill, 1983). For further investigations of CAD, and with a view to finding out the cellular location of this enzyme, a milder cell-disruption technique was sought.

There exists a wide-range of naturally-occurring exoenzymes whose function it is to degrade cell-walls and

described in this section was an attempt to isolate a naturally-occurring microorganism which secretes hydrolases capable of degrading the cell walls of Aspergillus terreus. If successful, a preparation of the exoenzymes would be made and employed to produce protoplasts of the mould on which more rigorous intracellular studies could then be performed.

A cell-wall preparation of Aspergillus terreus was prepared by harvesting mycelia from a 5-day fermenter culture and mixing with 1M NaOH at 37°C for 48h. This had the effect of dissolving or precipitating the majority of the mycelial constituents, whilst leaving the cell-wall polysaccharides essentially intact. This cell-wall preparation was then neutralized with 1M HCl, washed successively with water (3 changes), methanol (once) and water again (2 changes), and was then employed as the carbon source for a basal-salts culture medium. The basal medium contained the same constituents as those employed for the culture of Pseudomonas with the exception that the cell-wall preparation was used instead of the disodium itaconate. The medium was distributed in 100ml aliquots into 250ml conical flasks and autoclaved. Soil (10g), taken from a local wood, was used to inoculate such a flask which was then incubated on a rotary shaker at 25°C. After 48 hours, microscopic analysis of the contents of the flask revealed the presence of only a few different types of bacteria and

protozoa. A sample was taken and serial dilution of this was performed onto agar plates which had been prepared from the mycelial-wall basal medium (containing additionally 15g l^{-1} agar). Of the single colonies of microorganisms thus obtained, one particular type was shown to be readily capable of growth in the mycelial-wall flask cultures. This short, rod-shaped bacterium was apparently capable of secreting the full complement of enzymes required to hydrolyze completely the mycelial walls, and the effect was clearly visible under the microscope. Unfortunately, due to lack of time available, the potential of this organism for the possible production of fungal protoplasts has yet to be investigated.

GENERAL DISCUSSION

The existence of a wide variety of culture recipes for the production of itaconate by Aspergillus terreus serves to exemplify the complex array of factors which influence secondary metabolite production. What is ostensibly regarded as a reflection of the temperamental nature of metabolite overproduction is more accurately explained by our ignorance of the cellular processes which lead to it. Thus, the discovery that culture media which had previously been reported to support itaconate production in Aspergillus terreus did not do so under the conditions employed in this laboratory was intriguing but not surprising. A certain idiophasic response relies on the simultaneous effects of the correct nutritional environment and physiological state of the organism. The increasing acceptance of this view is highlighted by the growing number of publications which seek to explain "secondary metabolism and morphogenesis (changes in cellular structure) as correlated events in the overall process of cellular differentiation" (Bennet and Ciegler, 1982). As was discussed previously, investigators frequently attempt to reproduce the growth conditions for a fermentation whilst disregarding, for example, the method of production of the inoculum and hence the morphogenic state of the organism.

However, it is equally important to emphasise that even though the physiological response is governed by a varied series of regulators, the metabolic channel through which that particular response is propagated is unlikely to vary. Thus, the production of itaconate by Aspergillus terreus may be stimulated by a range of culture conditions, but the metabolic route by which this compound is synthesized will probably be the same in each case. Just as there should be no need to propose different pathways for the synthesis of, for example, the primary metabolite lactic acid, so the enzymic mode of synthesis of itaconic acid should be constant.

The simple requirement for the study of the enzymology of itaconate production in Aspergillus terreus is, therefore, a model fermentation system which stimulates this anabolic pathway. It is hoped that investigations at the enzymological level will eventually lead to an improved understanding of the above "cellular differentiation" mechanisms, and elucidate the connections between morphogenesis and secondary metabolism. In this way, investigators will be in a position to exercise more control over the process under examination and eventually, from a better understanding, make these processes more amenable to commercial exploitation.

In the model system employed for the studies reported

here, the yield of itaconate from the sucrose carbon source (the percentage ratio of the weight of acid produced to the weight of carbohydrate supplied) was between 25 and 30%. Although the yields quoted for the industrial production processes are frequently twice as much, this was regarded as a good system for further investigations in view of the fact that itaconate appeared to be the sole secondary metabolite (shown by paper chromatography). Furthermore, the yields quoted by other authors who have performed similar small-scale investigations are also in the region of 25-30% (Nowakowska-Waszczuk, 1973; Rychtera and Wase, 1981; Winskill, 1983). An additional advantage of this fermentation system was the convenience of the preparation of a control culture medium which did not support the production of itaconate, replacing the ammonium sulphate nitrogen source with potassium nitrate.

The fact that when nitrate is the nitrogen source little or no itaconate synthesis occurs was established in one of the earliest investigations of Aspergillus terreus (Moyer and Coghill, 1945). Nowakowska-Waszczuk (1973) suggested that this was probably related to the use of nitrate by the organism as a hydrogen-acceptor. However, this view was based on the belief that the organism lacked a functioning citric acid cycle and, therefore, also the type of electron transport chain usually associated with mitochondrial respiration. The

discovery, detailed in this report, of enzymes of the citric acid cycle in Aspergillus terreus effectively removes any reason for doubting the existence of normal eukaryotic electron transport and oxidative phosphorylation in this organism.

It is interesting to speculate that ammonia-dependent itaconate excretion is of the type proposed for other organic acids in Penicillium cyclopium by Roos and Luckner (1984). These authors observed a relationship between the coupling of H^+ and organic acid excretion with NH_4^+ uptake. In addition, citric acid production by Aspergillus niger has been shown to be associated with elevated intracellular levels of NH_4^+ ions which serve to permit unregulated flow of carbon through glycolysis by counteracting the natural feedback inhibition of phosphofructokinase by citrate (Kubicek and Rohr, 1981). Neither of these mechanisms has been observed in the presence of nitrate as the sole source of nitrogen, however, nor is there any direct evidence for their occurrence in ammonia-grown Aspergillus terreus.

The reported inability of Nowakowska-Waszczuk (1973) to find any evidence for citric acid cycle activity in mitochondria of Aspergillus terreus significantly affected the previously accepted view of itaconate synthesis. While many investigators saw the result as direct confirmation of the existence of an alternative

biosynthetic route, the opinion of the proponents of the CAD route was summarized by Miall (1978):

"...there seems no reason to postulate an entirely novel biosynthetic pathway in place of the one shown to operate in ...Aspergillus terreus (by Bentley and Thiessen, 1957). Surprisingly, what nobody has done is to attempt directly to demonstrate the presence or absence of the condensing enzyme (citrate synthase) in Aspergillus terreus mycelium making itaconic acid from carbohydrate... Unless this is done and the enzyme shown to be definitely absent, there seems little reason not to accept the conclusions of Bentley and Thiessen (1957)."

The demonstration, in this laboratory, that citrate synthase was, in fact, present in both itaconate-producing and non-itaconate-producing Aspergillus terreus was therefore of considerable significance.

Moreover, the simultaneous publication by Winskill (1983), in which the detection of citrate synthase and NADP-dependent dehydrogenase in Aspergillus terreus M490 was reported, was also in accordance with this proposal. Winskill (1983) also suggested that the failure of Nowakowska-Waszczuk (1973) to detect citric acid cycle activity was because of the difficulty of isolating intact mitochondria from

filamentous fungi. Indeed, the method employed by Winskill (1983) for the isolation of mitochondria (grinding with acid-washed sand) resulted in low respiratory control ratios and therefore is not a very satisfactory technique. The use of hydrolases to degrade the fungal cell walls and produce protoplasts should prove to be a more efficient method.

The radiolabelling experiments performed by Winskill (1983), in which the incorporation of ^{14}C-acetate into itaconate was investigated, also supported the view that citrate synthase, aconitase and CAD catalysed successive steps to itaconate. The results of previous radiolabelling experiments (Corzo and Tatum, 1953; Bentley and Thiessen, 1957), whilst supporting this proposal, could also have been interpreted as indicating itaconate synthesis via the condensation of three molecules of acetate to form 1,2,3, tricarboxypropane (Shimi and Nour El Dein, 1962; Nowakowska-Waszczuk, 1973). The results of Winskill (1983) were less equivocal.

Winskill (1983) had, therefore, gone a long way to abrogating the results of Nowakowska-Waszczuk (1973) and had furthered the work of Bentley and Thiessen (1957) in providing more evidence for the existence of CAD. However, there were elements of the findings of Winskill (1983) which were in disagreement with the results

produced in this laboratory. For example, although the values of the specific activity of NADP-dependent isocitrate dehydrogenase are in the same order of magnitude, the specific activity of citrate synthase detected by Winskill (1983) is approximately ten-fold greater than that detected in this laboratory. In addition, as was discussed earlier, the decrease in isocitrate dehydrogenase activity reported by Winskill (1983) was not observed in this laboratory. Such discrepancies may possibly be attributed to strain differences; Aspergillus terreus M490 was used in the studies of Winskill (1983). Finally, emphasising the similarity of the two sets of findings, the criterion proposed by Miall (1978) for confirmation of itaconate synthesis via citrate and cis-aconitate appears to have been satisfied.

Following the publication of the results of Winskill (1983), and the concomitant discovery of various citric acid cycle enzymes, detailed in this report, it was considered desirable to investigate more closely the enzymic formation of itaconate in Aspergillus terreus. The enzyme-linked spectrophotometric assay for itaconate, developed accordingly, has proved to be effective in monitoring itaconate formation during both the fermentation of Aspergillus terreus and the enzymic decarboxylation of cis-aconitate. Previously, the most frequently employed procedure for the quantitative

determination of itaconate was that devised by Friedkin (1945). The latter method uses a 'Bromine Reagent' solution which is buffered at pH 1.2 in order to ensure the selective absorption of bromine by the itaconate in the sample. Following the reaction of bromine with itaconate in a sealed iodine flask, a solution of potassium iodide is added. The excess unreacted bromine displaces the iodide and thus forms free iodine which is then quantitated by titration against sodium thiosulphate with starch as the indicator. The assay is effective with samples containing between 0.1 and 1mmole of itaconate. Larsen (1957) devised a semi-micro modification of this method which may be used to assay itaconate down to the lower limit of 5µmoles. However, in spite of its widespread usage for the determinations of itaconate present in culture media of Aspergillus terreus, this assay is clearly not amenable to the investigation of itaconate formation at an enzymological level. The time-consuming nature and relative insensitivity of the assay severely restrict any alternative applications. A further modification of this assay involving spectrophotometric detection of bromine decolourization (detailed in Materials and Methods) has been devised by Adler (1957). This assay is effective over the range of 0.1 to 1µmole of itaconate and is far simpler to perform than the iodometric titration, but is relatively inaccurate because of the sensitivity of the 'Bromine Reagent' to light. No details have been

published on the properties of the other methods of itaconate detection, which are gas-liquid chromatography (Tabuchi et al., 1975) and isotachophoresis (Horitzu et al., 1983).

Results presented in this report have shown the enzyme-linked spectrophotometric method of itaconate determination to be effective over the range 1 to 100nmoles of itaconate. From the standard curve (Fig. 2), a change in absorbance of 0.15 units was produced by 13nmoles of itaconate. A standard curve for the assay of itaconate using the bromination method adapted for spectrophotometry was also constructed and the same change in absorbance was produced by 500nmoles of itaconate. The enzyme-linked assay was, therefore, 40 times more sensitive than the spectrophotometric bromination method. The specificity of the assay is attained by the action of the three enzymes involved, and precision is conferred by the large absorption coefficient of pyruvate phenylhydrazone. It is possible that interference may arise if the test solutions contain compounds possessing carbonyl groups capable of reacting with phenylhydrazine. Under such circumstances the assay may be modified to follow pyruvate formation more specifically by using lactate dehydrogenase and NADH. However, no such difficulty was encountered when the assay was used to follow the fermentative production of itaconate. This assay for itaconate is, therefore, more

sensitive and accurate than previously reported methods. It is rapid and convenient to perform; it requires no specialized equipment; apparently, any species of Pseudomonas which is capable of growth on itaconate may be employed; finally, its most advantageous feature lies in the fact that it is performed under physiological conditions and may therefore be used to follow the enzymic production of itaconate continuously.

The modification of the enzyme-linked assay, in which an isocitrate lyase-deficient mutant of Pseudomonas aeruginosa was employed, led subsequently to confirmation of the presence of CAD in cell-free extracts of itaconate-producing Aspergillus terreus. No CAD activity was detected in mycelia prior to the production of itaconate, nor in mycelia which were cultured under conditions which did not support the production of itaconate. When seen in the light of the results of Winskill (1983) and of those produced earlier in this laboratory, all of which have been discussed above, the evidence against the existence of CAD has been largely countered. Moreover, this new method by which the decarboxylase has been detected and assayed has the additional advantage of being more effective than the previously employed manometric procedure. Bentley and Thiessen (1957) measured CO_2 evolution as a function of enzyme activity, whereas the assay described here measures itaconate formation directly. In addition, the

enzyme-linked assay has proved to be more sensitive in the detection of CAD activity and more flexible in scrutinizing different characteristics of the enzyme.

Of further significance to this work is the fact that the manometric detection of CAD activity has also been performed independently by another group of investigators. In work which appears to have gone unnoticed by other authors, Pal and Krishnan (1959, 1961 and 1964) confirmed the results of Bentley and Thiessen (1957), and used the manometric assay for CAD to perform further studies on the enzyme. In common with the results obtained in this laboratory, Pal and Krishnan (1961) observed the precipitation of CAD between 40% and 60% ammonium sulphate saturation. Furthermore, these authors determined the pH optimum of the enzyme to be 5.8; the values of 5.6 obtained by Bentley and Thiessen (1957) and 5.5 (this report) are in close agreement with this. However, there is a significant difference between the value obtained for the Km of CAD for cis-aconitate of 0.15mM, reported here, and that determined by Pal and Krishnan (1961) of 5mM, but the narrow range of substrate concentrations used by the latter authors (1-8mM) probably led to a large error. Clearly, when presented with all the evidence for the occurrence of CAD, it would be unnecessary to propose the existence of an alternative pathway for the formation of itaconate in Aspergillus terreus.

The discovery of the Km of CAD for cis-aconitate to be 0.15mM is interesting. Glusker (1971) has reported the Km of aconitases (from a wide variety of sources) for

cis-aconitate to be in the region of 0.1-1mM, whereas the Km values for citrate and isocitrate are frequently 10 times higher. If the Km of Aspergillus terreus aconitase for cis-aconitate were found to lie in the same region as that of other aconitases, a situation could be envisaged in itaconate-producing Aspergillus terreus where CAD competes successfully with aconitase for the intracellular cis-aconitate. Thus, the appearance of CAD in the cell may itself be sufficient to regulate the production of itaconate, in which case other mechanisms such as a reduction in isocitrate dehydrogenase activity need not occur. However, such a proposal is purely speculative and other factors, such as the relative specific activities and V_{max} values of CAD and aconitase, would also need to be taken into consideration.

With the use of the 3-litre fermenter, the details of the itaconate fermentation were scrutinised more closely. The demonstration of the presence of more than sufficient CAD activity to account for the amount of itaconate produced per unit time was a further indication that itaconate is synthesized via the decarboxylation of cis-aconitate. Furthermore, the absence of any apparent significant changes in the activities of citrate synthase, aconitase and NADP-dependent isocitrate dehydrogenase indicated the possible regulatory importance of the appearance of CAD at the onset of acidogenesis. Moreover, the

demonstration that itaconate production coincided precisely with the appearance of intracellular CAD activity emphasises the central role played by this enzyme in itaconate production. However, the simplistic nature of these observations and consequent conclusions tends to conceal the complexity of the physiological changes which occur in the organism at the start of the idiophase.

The importance of the dual consideration of the morphogenic state of the organism and its nutritional environment to secondary metabolism was discussed briefly at the beginning of this section. As one aspect of the latter of these considerations, the levels of carbohydrate, ammonia and phosphate were monitored throughout the fermentation. The depletion of phosphate from the culture medium was shown to coincide precisely with the onset of itaconate production and, therefore, with the rise in CAD activity. Carbohydrate and ammonia were shown to be present in excess during the whole course of the fermentation. However, this observation is not new. The fact that phosphate depletion from the medium coincided with the onset of itaconate production was first reported by Rychtera and Wase (1981) and, from a survey of many of the media which have been employed for itaconate production, it appears that phosphate was the growth-limiting nutrient in each case. The effect of a nitrogen-limited culture on the production of itaconate

has yet to be reported, but from the phosphate-pulse experiment, in which nitrogen eventually became a limiting nutrient, the preliminary indications are that such a condition is unfavourable to the production of itaconate.

The conclusions that can be drawn from the phosphate-pulse experiment (Fig. 25) are limited. The speed with which the added phosphate was absorbed by the mycelium precluded an unequivocal answer to whether the effects of phosphate depletion were in this way reversible. That the organism takes up extracellular phosphate very rapidly had also been observed in previous fermentations. Phosphate present in the culture medium was completely absorbed in the early stage of the fermentation and before the amount of biomass had reached half of its final level (see, e.g., Fig. 24). Presumably, when there was insufficient intracellular phosphate to fulfill all of the metabolic requirements for exponential growth, central metabolism became affected in such a way that the culture progressed from tropophase to idiophase.

It appeared in the phosphate-pulse experiment that the supplementary phosphate was added before the start of the idiophase. However, although the organism was able, under these conditions, to produce more biomass, the intracellular mechanism triggering CAD activity had already been activated. This tends to suggest that the

effects of phosphate depletion could not be reversed by addition of more phosphate. On the other hand, although the detected activity of CAD appeared to be unaffected by the phosphate pulse, there was also an indication that the initial rate of the production of itaconate was lower than in previous pulse-free fermentations (as indicated in Fig. 25). This observation may be related to the lower activity of CAD obtained in phosphate buffer compared to that obtained in triethanolamine buffer. If, from further experiments, CAD was shown conclusively to be inhibited by phosphate, an in vivo regulation of CAD by intracellular phosphate could be envisaged.

Clearly, further investigations of the effects of phosphate, at both a physiological and an enzymological level, have to be performed. If the fermentation was found to be amenable to chemostat studies, then these investigations are likely to be more conclusive.

The observation that intracellular CAD activity fell rapidly on depletion of ammonia from the medium is also interesting. Once again more investigations will have to be performed, but it may be speculated (as before) that ammonia is closely associated with the maintenance of the intracellular pH. On the depletion of ammonia from the medium, the balance between ammonium ion uptake and proton and organic acid expulsion may be disrupted. However, such a proposal does not apply to the production

of citric acid by Aspergillus niger since this process can be initiated by ammonia depletion from the medium as well as by phosphate depletion (Kubicek and Rohr, 1981).

CONCLUSIONS AND NEW PERSPECTIVES

At the beginning of the preceding section, the general dearth of knowledge associated with the physiology of secondary metabolite production was indicated. At the end, a typical example was provided. However, the intervening paragraphs also included some evidence of progress. Thus Aspergillus terreus has been shown to possess many of the citric acid cycle enzymes; there appeared to be no gross differences in citric acid cycle enzyme activity between the itaconate-producing and non-itaconate-producing organism. A new enzyme-linked assay for itaconate has been devised which has a greater application and flexibility than previously-described assays. Furthermore, the assay has been shown to be applicable to the detection and determination of CAD, the presence of which has been confirmed in itaconate-producing mycelia of Aspergillus terreus. No CAD activity was detected in mycelia prior to the production of itaconate, nor in mycelia cultured under conditions not supporting the production of itaconate. This, and the correlation of enzyme activity and acid production have led to the conclusion that itaconate production in Aspergillus terreus occurs by the decarboxylation of cis-aconitate. Furthermore, the depletion of phosphate from the culture medium has been shown to be a possible stimulator of an increase in intracellular CAD activity and concomitant acidogenesis.

CAD has also been shown to have a Km for cis-aconitate of 0.15mM, and a pH optimum of 5.5.

There is a tendency among reviewers to unify the phenomenon of organic acid production by microorganisms. However, it must be realised that there is a clear difference between the production of organic acids which are primary products of metabolism (e.g. citrate, fumarate, malate, gluconate and lactate) and those which are synthesized exclusively in the idiophase (e.g. itaconate). At the same time, itaconate is hardly a close relation of the other well-known secondary metabolites of fungi (e.g. terpenes, steroids, polyketides and derivatives of complex fatty acids). Therefore investigators frequently treat the production of itaconate by Aspergillus terreus in isolation of other such phenomena and then proceed to correlate such processes only when connections are obvious. The same approach has been taken in this report in which most of the comparisons have been made with citric acid production by Aspergillus niger. This is primarily because of the similarity of the organisms involved and the structures of the acids, and because of the comprehensive studies of the biochemistry of citric acid production which have been performed.

The end result of the work described in this report appears to have been the disclosure of more areas of

ignorance than of a general enlightenment. However, at least some of the methods by which further investigation may take place have been revealed. Thus there should no longer be controversy surrounding the route of itaconate production in Aspergillus terreus, and the new assays for itaconate and CAD should contribute significantly to future studies. It will be interesting to investigate the intracellular location of CAD and especially in relation to the location of citrate synthase, aconitase and isocitrate dehydrogenase. Pal and Krishnan (1964) proposed that CAD occurs exclusively in the cytosol and Mattey (1977) found no evidence for citrate synthase or aconitase activity outside the mitochondria of Aspergillus niger. Preparation of fungal protoplasts, possibly by using bacterial exoenzymes as discussed earlier in this report, may help to clarify the situation in itaconate-producing Aspergillus terreus.
Determination of the Km of Aspergillus aconitase for cis-aconitate might lead to elucidation of the regulatory significance of CAD in itaconate production. Furthermore, purification of CAD should help to investigate possible metabolic control mechanisms of the enzyme such as, for example, phosphorylation/ dephosphorylation.
Alternatively, it may be discovered that the enzyme activity is controlled primarily at the level of transcription, where phosphate depletion might activate the gene for CAD. Purification of CAD could also prepare the way for experiments on the immobilization or

co-immobilization of the enzyme. Recently, the first report of itaconic acid production by Aspergillus terreus immobilized in polyacrylamide gels appeared (Horitzu et al.,1983); monitoring the CAD activity in this whole-cell immobilization process could be useful.

The discovery that itaconate synthesis is only one enzyme removed from central, primary, metabolism may be an indication of the relatively minor genetic difference between an itaconate-producing and a non-itaconate-producing organism. Thus, it may be possible to clone the gene for CAD into a bacterial or yeast cell which is more amenable to large-scale culture and subsequent product recovery. In this sense, the new assay, described here, for itaconate may prove to be useful in the development of a rapid screening procedure for itaconate-producing organisms.

In order to obtain more conclusive results on the physiology of the process and, more especially, the combined effects of nitrogen and phosphate, chemostat studies of the kind recently reported by Kristiansen et al. (1982) on citric acid production by Aspergillus niger will have to be performed. More importantly, such experiments may lead to the elucidation of the reasons for the cessation of itaconate production. As could be seen from Fig. 24, acidogenesis ended in spite of the presence of significant intracellular CAD activity. It

therefore appears that other factors, possibly transmembrane gradients or inactivation of an enzyme other than CAD, were responsible. Successful identification of these factors could eventually prove useful in improving industrial yields of itaconate.

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